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THE EFFECT OF VASOACTIVE INTESTINAL PEPTIDE ON CHLORIDE CONDUCTANCE IN AIRWAY EPITHELIAL CELLS

Shelagh Anne Houghton

Yale University

1997

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THE EFFECT OF VASOACTIVE INTESTINAL PEPTIDE ON CHLORIDE CONDUCTANCE IN AIRWAY EPITHELIAL CELLS

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Shelagh Anne Houghton

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THE EFFECT OF VASOACTIVE INTESTINAL PEPTIDE ON CHLORIDE CONDUCTANCE IN AIRWAY EPITHELIAL CELLS.

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The aim of this study was to characterize the effect and describe the mechanism of action of vasoactive intestinal peptide (VIP) on chloride conductance in tracheal epithelial cells.

Two established cell lines were examined using single channel inside-out excised patch clamping technique: the IB3-1 line, a cell line from a cystic fibrosis (CF)-affected child, and the S9 line, obtained by transfection of the IB3-1 line with wild-type cystic fibrosis transmembrane conductance regulator (CFTR). All cells were pretreated with VIP (1μM) for ten minutes and then examined in both cell-attached and excised patch mode. If channel rundown occurred in the excised mode, ATP (1mM) was added to the bathing solution.

In the IB3-1 line, 69% (9/13) of patches demonstrated no channel activity. The remaining four patches (31%) contained channels characterized by a large amplitude and activation only after depolarizing voltages ≥ +70mV. Both attributes are consistent with outwardly rectifying chloride channel (ORCC) activity. These channels displayed no rundown and were not affected by addition of ATP.

In the S9 cell line, 83% (10/12) of excised patches demonstrated channel activity consistent with CFTR chloride channels. Mean conductance was 10.4pS±0.39pS. Channel activity ran down in 80% (8/10) of these channels; 75% (3/4) of patches treated with ATP after rundown demonstrated restoration of channel activity. In addition, 50% (5/10) of S9 patches with channels also demonstrated a larger channel suggestive of ORCC.

This study strongly suggests that VIP increases chloride conductance by stimulation of CFTR chloride channels in the apical membrane. VIP may activate CFTR directly, or indirectly through the second messenger cAMP. The data supports potential therapies that would maximize or complement cAMP-stimulated chloride current, particularly in patients with CF mutations productive of suboptimally functioning CFTR.

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1. Introduction

1-1. Inflammation

Inflammation is fundamentally a protective response initiated against substances or organisms that threaten the body's homeostatic mechanisms. The inflammatory response begins when cellular injury occurs by a variety of insults, including microbes, toxins and mechanical stress. It continues even when the offending agent is eliminated, as the host attempts to repair necrotic tissue and remove cellular debris. However, in both normal and pathophysiology, the inflammatory response can extend beyond the scope of constructive repair, and, in fact, cause tissue damage with exacerbation or development of symptoms unrelated to the initial condition.

The basic sequence of events in the acute inflammatory response is similar against many forms of injury. Immediately following tissue injury, major changes occur in blood vessels supplying the violated area which permit both increased blood flow and transmigration of leukocytes from the blood to the interstitium. Vessels become dilated and increasingly permeable. This leads to slowing of the circulation as protein-rich fluid flows into the interstitium, leaving predominantly red blood cells in vessels. With stasis, leukocytes extravasate, first adhering to the endothelium and then squeezing through junctions between endothelial cells into the interstitial space. Having escaped the confines of the vasculature, leukocytes move toward the site of injury by chemotaxis and, having reached the appropriate site, are activated by distinct chemical and mechanical signals. The leukocytic response to membrane changes is manifest as release of

lysosomal enzymes, oxygen metabolites, and products of arachidonic acid metabolism (prostaglandins and leukotrienes). These products are essential mediators of the inflammatory response and form part of the amplification cascade following acute injury. However, unlike cellular immunity, these defenses are non-specific and can paradoxically cause direct endothelial damage and tissue injury. When this response proceeds unchecked, the inflammatory response itself engenders a milieu within the affected system characterized by edema, poor perfusion, fibrosis, and creation of an environment suitable for superimposed bacterial or fungal infection.

Signaling required for the necessary vasodilation, increased permeability, and chemotaxis stems from one of many distinct inflammatory mediators, which may be derived from damaged cells, plasma, mast cells, and nerve endings. Cells stimulated by mechanical or chemical disruption are the source of arachidonic acid metabolites, the prostaglandins and leukotrienes. Activated plasma triggers four interrelated cascades: the complement, kinin, fibrinogen, and coagulation systems. Activated mast cells and nerve endings of both the autonomic and non-adrenergic, non-cholinergic systems produce neuropeptides, such as VIP, Substance P, and the tachykinins. How these inflammatory mediators modulate function specifically in the human respiratory tract is important in understanding both the etiology and possible therapeutic options in lung pathophysiology.

1-2. Physiologic Role of Inflammation in the Lung

In the respiratory system proper functioning of the inflammatory response prevents chronic infection or colonization by pathogens and eradicates chemical or

mechanical irritants. The epithelial layer lining the respiratory tract is an integral part of the defense system. Humans rely on the mechanical barrier of the nasal mucosal layer, immunologic systems in the conducting airways and alveoli, and the mucociliary ladder (Welsh, 1988).

Airway secretions provide the essential conduit of the mucociliary clearance ladder. The ladder serves mechanically to remove inhaled matter from the lung through a combination of ciliary movement and fluid wash. Cilia are immersed in a 6 μm-thick layer of fluid, the *aqueous sol* or *periciliary* layer. Quantity and quality of the fluid layer are controlled by electrolyte transport through the respiratory epithelium. The mucus layer, which is 5 to 10 μm thick, covers the periciliary layer, and traps foreign particulate matter. Mucus is produced primarily by non-ciliated goblet cells and also, in the larger conducting airways, by submucosal glands. Beating of the cilia in the periciliary fluid establishes coordinated movement by which foreign matter trapped in the mucus layer is transported toward the glottis (Welsh, 1988).

Inflammatory mediators promote changes in mucus production, vascular permeability, smooth muscle contractility, and fluid and ion movement that collectively enhance both recruitment of opsonic and phagocytic elements into disrupted areas and clearance of noxious material from the respiratory tract along the mucociliary ladder. The normal response to infection or irritation consists of release of mediators that stimulate increased secretions into the airway lumen to wash out irritants and supply phagocytic elements. These secretions can be too voluminous, as in asthma, which leads to airway plugging, or insufficient, as in cystic fibrosis, with consequent hyperviscosity

and sludging. In either case, bacteria and airway irritants persist. In addition, the ongoing nidus of irritation prompts continuous inflow of host cellular elements (PMN's, lymphocytes, and macrophages) which, as described above, can cause direct damage. The cellular mechanisms by which inflammatory mediators such as leukotrienes, prostaglandins, VIP, Substance P, and the tackykinins increase airway secretions remain unclear. Understanding the details of these pathways may ultimately provide therapeutic options in the treatment of severe respiratory diseases.

1-3. Regulation of Airway Secretions

Both chloride secretion and sodium absorption influence the composition and quantity of the fluid bathing the respiratory epithelium. The relative contribution of either of these functions is determined by the neurohormonal milieu and the anatomical region of the airway (McCann and Welsh, 1990). An ability collectively to secrete fluid depends on the structure of each epithelial cell lining a particular portion of the airway. Cells are polarized, with an apical aspect lining the lumen, and a basolateral face lining the interstitial space. Membrane at either end has properties specific to the region of the respiratory system and reflects the physiological role of the cell. The isolation of certain transporters or channels to the apical or the basolateral pole permits either net absorption or net secretion. Epithelial cells lining the respiratory tract are joined along their apical (lining the lumen) borders by tight junctions. The resulting wall of cells creates a semi-permeable barrier: ions, certain solutes, and water may move across the barrier, while other molecules and charged particles may not pass. Two pathways exist by which solutes may move: through the tight junctions and between cells, referred to as the

paracellular pathway, or through channels or transporters in the apical membrane of the cells themselves, also known as the *cellular pathway* (Welsh, 1988).

The balance between sodium absorption and chloride secretion from the apical membrane of respiratory epithelium is the primary factor in determining the degree of fluid production. Differences in the magnitude of chloride secretion and net luminal fluid flow in various parts of the human airways depend on the prevalence of chloride channels correlating with the functional demands of the cells in the region. The mechanism of chloride secretion is otherwise the same (Figure 1). First, chloride enters cells in a neutral cotransport with sodium and potassium along the basolateral membrane against an electrochemical gradient (more intracellular than extracellular chloride). The nature of this cotransporter may differ among cell types, but is likely to be either a Na^+/Cl^- cotransporter or a $Na^+/ K^+/Cl^-$ cotransporter (McCann and Welsh, 1990). Energy necessary for this process is provided by coupling with sodium, which is moving down an electrochemical gradient created by the Na^+,K^+-ATPase also located in the basolateral membrane. The basolateral membrane has potassium channels, which permit passive movement of potassium down the chemical gradient created by both the Na^+,K^+-ATPase and the chloride transporter. The potassium channels are important in maintaining negative intracellular voltage (Welsh, 1988).

The second step in chloride secretion through the cellular pathway involves specific chloride channels located in the apical membrane. Several distinct chloride channels have been identified in the apical membrane of both epithelial and nonepithelial mammalian cells. These channels, which include the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, an outwardly rectifying chloride channel

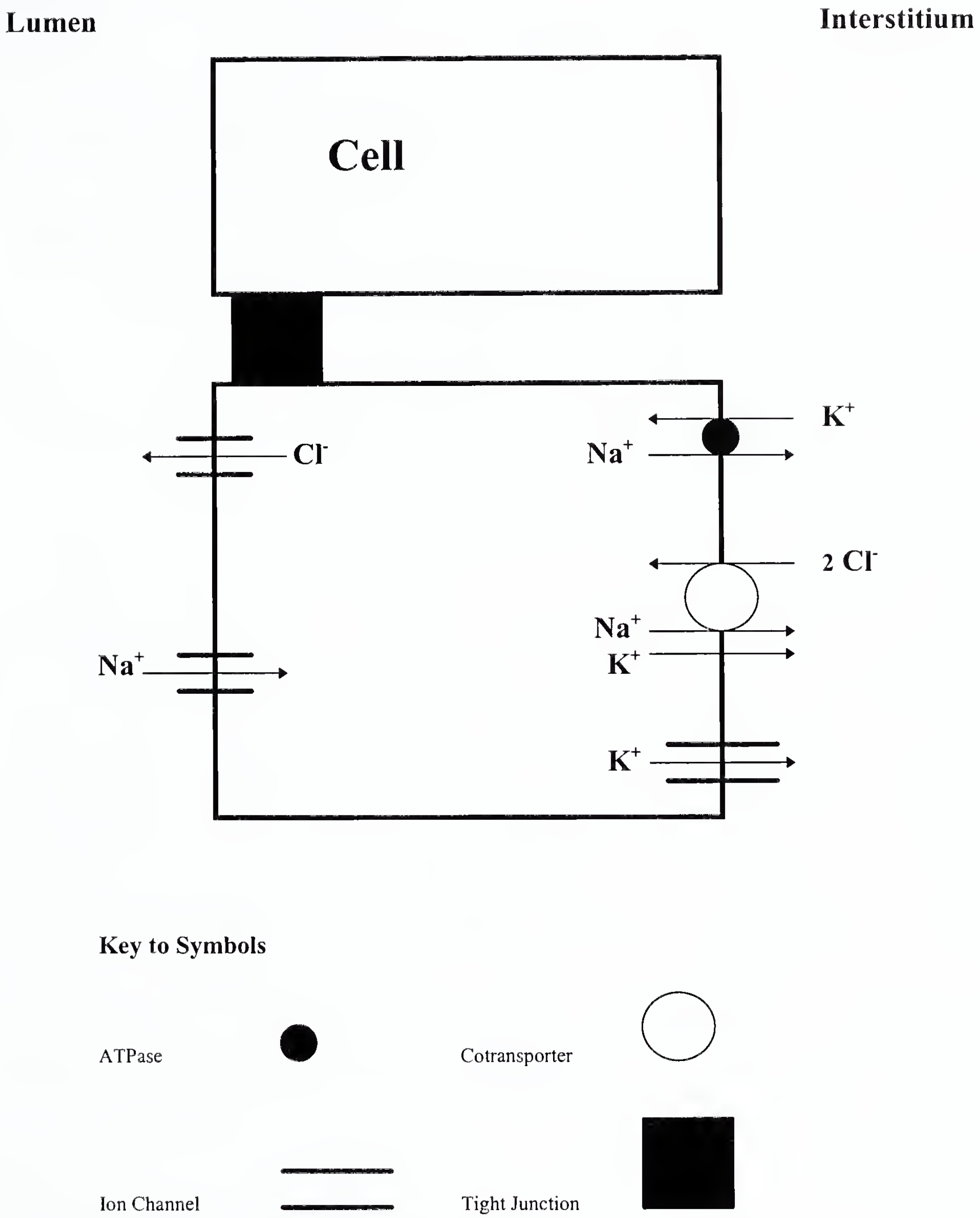


Figure 1. Mechanism of chloride secretion by airway epithelial cells. The basolateral membrane contains a Na^+/Cl^- or a $Na^+/K^+/Cl^-$ cotransporter, the Na^+,K^+ ATPase, and a K^+ channel. The cotransporter exploits the Na^+ gradient generated by the ATPase to accumulate chloride within the cell. Opening of a channel in the apical membrane permits efflux of chloride down its electrochemical gradient. In a non-leaky epithelium, K^+ leaves the cell on the basolateral side to compensate for the charge lost by the cell in the secretion of chloride.

(ORCC), and a calcium-activated chloride channel, are responsible for the movement of chloride from the serosal to the mucosal side of the cell. Fruitful functional studies have utilized apical membrane, whole-cell, and single channel patch clamp techniques to measure conductance and voltage variability. Essential to distinguishing chloride channels is examination of the individual functional profile. Average chloride conductance, current-voltage (I-V) relationship, voltage dependence, and reaction to agonists or antagonists comprise a set of features apparently unique to each variety of channel.

1-3.1. CFTR Chloride Channel

In the early 1980's electrophysiologic studies of cystic fibrosis (CF) affected sweat ducts (Quinton, 1986) and airways (Knowles et al., 1983) demonstrated that the primary defect in CF was a decrease in chloride permeability in epithelial cells. In 1989, the genetic mutation associated with CF was identified. The gene that is defective in CF encodes for the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989)(Bear et al., 1992). Mutations in CFTR eliminate or reduce chloride conductance from the apical membrane of predominantly epithelial cells and, clinically, result in the phenotype characteristic of cystic fibrosis, the most common lethal genetic disease in Caucasians. Unclear, however, was the issue of whether the physiologic defect resided with an improperly functioning channel or with defective regulation of a normal channel. Conjecture regarding the role of CFTR revolved around two primary options: either CFTR was itself a chloride channel or CFTR was a regulator of chloride, and perhaps other, ionic channels.

Prior to identification of CFTR, excised, inside-out single channel patch studies suggested a link between protein kinase A (PKA) and intracellular nucleotides, and activation of chloride channels. While addition of the catalytic subunit of PKA and ATP opened chloride channels in membranes from normal cells, similar manipulation of CF-affected cells failed to increase chloride movement. This data supported early theories that the CF defect presented a problem distal to the receptor or initiation of a second messenger cascade, perhaps residing in a regulatory protein or the channel itself (Schoumacher et al., 1987)(Greger et al., 1988)(Li et al., 1988). However, identification of CFTR was necessary to complete the puzzle.

The structure of the protein predicted by the amino acid sequence consists of two sets of six transmembrane domains, two nucleotide binding folds, and an intracellular hydrophobic regulatory, or R, domain (Figure 2). This structure resembles that of the ATP binding cassette, or ABC, family of transporters, a group of proteins that mediate selective movement of solutes across membranes (Higgins, 1995). Among ABC transporters are the P-glycoproteins, which transport verapamil and vincristine and confer drug resistance in bacteria and cancer cells; the SUR receptor for sulphonylureas; and the sterile 6 protein of yeast which transports the “a” mating factor (Higgins, 1995). All ABC transporters share a common structure, with four fused or independent core domains. Two membrane spanning domains straddle the core and serve as the pathway through which solute crosses the membrane; these transmembrane domains determine the specificity of the channel by their particular amino acid sequence. Alteration of the anion selectivity after mutation of four basic amino acids in the CFTR transmembrane domain justified belief that CFTR itself is a chloride channel (Anderson et al., 1991b). In

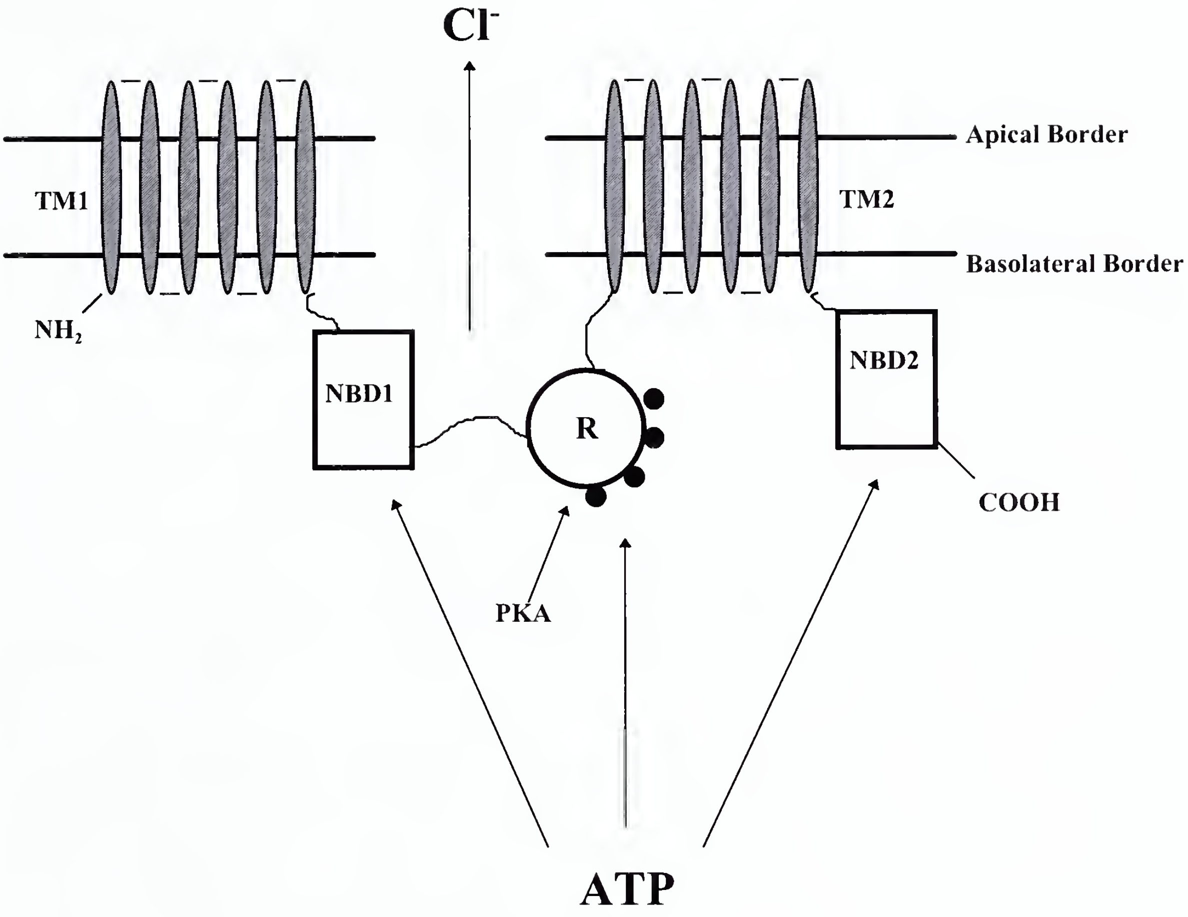


Figure 2. Cystic fibrosis transmembrane conductance regulator. The structure, as predicted by the amino acid sequence, consists of two membrane-spanning domains (TM) with six membrane spanning segments each, two nucleotide binding domains (NBD), and an intracellular regulatory domain (R). The R domain contains multiple consensus phosphorylation sites for priming of the channel by PKA and PKC (denoted by ●).

addition, two putative nucleotide-binding domains on the cytosolic surface provide energy for solute movement via ATP hydrolysis in many ABC transporters (Hyde et al., 1990)(Ames and Lecar, 1992)(Higgins, 1995). The role of these domains in CFTR is discussed below.

The CFTR chloride channel is singular among ABC transporters in that it is an ion channel, not an active transporter. In addition to being a channel itself, CFTR also regulates several other ion channels, including the outwardly rectifying chloride channel (see discussion below); sodium channels, with increased sodium absorption through dysfunctional regulation by CFTR of the amiloride-sensitive sodium channel, ENaC (Chinet et al., 1994); and calcium-activated chloride channels, which are up-regulated in cystic fibrosis (Grubb et al., 1994). Consequent derangement of these other channels may contribute to the pathophysiology of CF.

Also unique to CFTR among ABC transporters is the regulatory, "R" domain, which contains multiple phosphorylation sites for cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) (Riordan et al., 1989)(Rich et al., 1991). The current working model for CFTR pictures the protein forming a chloride channel in which the transmembrane domains form the pore and the R domain functions as a channel plug, somewhat like a ball fitting into the pore. Phosphorylation of the R domain by PKA prepares the protein, either by effecting a conformational change or by creating an electrostatic repulsion of the R domain away from the membrane, to be accessible to a chloride ion (Cheng et al., 1991). When the R domain blockade is removed, binding of ATP at the first NBD and subsequent hydrolysis permits channel opening. Binding and hydrolysis of ATP at the second NBD allows channel closure. Dephosphorylation of the

R domain by specific phosphatases results in actual closure of the channel (Tabcharani et al., 1991)(Berger et al., 1993)(Hwang et al., 1993)(Davis et al., 1996). Evidence to support this current model will be discussed in subsequent sections.

As described, the CFTR channel is regulated by PKA phosphorylation and intracellular ATP. Agents that increase cAMP, such as B-adrenergic agonists, forskolin (an adenylate cyclase activator), and 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor), effectively increase apical chloride conductance by initiating the second messenger cascade leading to activation of PKA. Electrophysiological studies have confirmed the unique functional characteristics of this channel. At baseline, the CFTR channel exhibits little or no chloride conductance; addition of cAMP results in a dramatic increase in chloride current (Anderson et al., 1992). The channel is not affected by intracellular calcium; the response to cAMP persists in cells which have been buffered with calcium chelators, such as EGTA. The CFTR chloride channel is a linear, 8-10pS chloride channel located in the apical membrane (Cliff et al., 1992) and lacks time-dependent voltage effects (Anderson et al., 1991c). The channel is further characterized as being selective for anions over cations, and by having an anion selectivity preference of bromide>chloride>iodide>fluoride (Anderson and Welsh, 1991). Slight outward rectification seen with high pipette chloride concentration is most likely secondary to the pipette-to-cell chloride gradient, and is not of physiologic consequence (Cliff et al., 1992). Physiologically, it is classified as non-rectifying (Overholt et al., 1995). This channel is inhibited by glibenclamide, which was previously thought to be a specific K^+ channel blocker (Rabe et al., 1995).

1-3.1.1. cAMP Dependence of CFTR

Studies using recombinant CFTR to transfect both CF-affected cells and normal cells that do not ordinarily express CFTR have demonstrated the role of cAMP in channel activation. Whole cell patch clamp studies using CF airway epithelia and a CF pancreatic cell line (CFPAC-1) demonstrated an increase in chloride conductance after transfection with CFTR (Anderson et al., 1991c) (Drumm and Collins, 1993). Additional studies in which nonepithelial mammalian cells without endogenous CFTR were transfected with recombinant CFTR demonstrated generation of chloride current activated by cAMP agonists including forskolin, IBMX, and dccAMP (a membrane-permeable form of cAMP). Cell lines employed included NIH/3T3 fibroblasts (Anderson et al., 1991b), Chinese hamster oocytes (CHO) (Anderson et al., 1991c), and HeLa cells (Anderson et al., 1991c). When these same cell lines were transfected with mutant CFTR vector (ΔF508, deletion of phenylalanine at position 508, the most common mutation), no increase in chloride conductance was observed upon stimulation. Moreover, when T84 cells, human colon carcinoma epithelial cells with high endogenous expression of CFTR, were transfected with CFTR, cAMP-activated halide permeability was greater than that observed with non-transfected cell lines. This indicates that overexpression of CFTR causes an increased anion permeability, and supports the suggestion that CFTR effects a dose-related response in cAMP-mediated anion conductance (Anderson et al., 1991c). Similar single channel studies have produced data with results supporting the whole cell studies linking CFTR to a reversible cAMP-mediated increase in chloride current (Bear et al., 1991)(Dalemans et al., 1991)(Kartner et al., 1991)(Anderson et al., 1992).

Cells expressing endogenous CFTR display cAMP-activated chloride conductance with functional properties similar to those described in cells transfected with known CFTR. In order for a chloride current to contribute to the net movement of fluid, the current must originate in the apical membrane of the cell. To analyze apical membrane chloride movement specifically, normal cell lines were treated with nystatin, which functionally eliminates the basolateral membrane, and then grown on permeable supports. In this setup, normal airway and intestinal epithelium (T-84, Caco-2, and HT-29) displayed an increase in apical membrane chloride conductance upon addition of cAMP. In contrast, epithelial cells cultured from CF-affected airways did not demonstrate an increase in chloride conductance with addition of cAMP (Anderson and Welsh, 1991). Experiments utilizing the Caco-2 cell line transfected with a mutant regulatory subunit of cAMP-dependent protein kinase have shown a decreased chloride efflux in response to potent cAMP agonists, including forskolin, membrane permeable analogs of cAMP, VIP, PGE_2, and isoproterenol. These results indicate that cAMP and PKA are essential components of secretogue-regulated chloride conductance (Krolczyk et al., 1995). The similarities between the functional characteristics of chloride channels observed in these studies of the apical membrane of normal and CF-affected cells compared with the properties of channels seen in CFTR-transfected cells is strong evidence that the cAMP-regulated chloride channel observed in the apical membrane of natural epithelium operates through CFTR.

Cells expressing [illegible] coexpression with functional [illegible] ligand TGF-β. In order to [illegible] the [illegible] [illegible]

1-3.1.2. Role of the Regulatory Domain

Other studies have further elucidated the mechanism of action of PKA phosphorylation and ATP binding and hydrolysis, creating a picture of the integrated functioning of the R domain and nucleotide binding domains. One model posits the R domain as a structural inhibitor, which physically blocks the channel. This theory is supported by studies in which CFTR with a deleted R domain functions exactly as a wild-type CFTR chloride channel except that it is constitutively open (Rich et al., 1991). In wild-type CFTR, one of four serine residues in the R domain must be phosphorylated for channel activation (Cheng et al., 1991). Although data had suggested that simultaneous mutation of all four serine residues was required to prevent cAMP-dependent channel activation, recent studies demonstrate that mutant CFTR lacking all four serine residues can still be phosphorylated to an active chloride channel, but that the open state probability is reduced (Rich et al., 1993). The implication that other regulatory sites within the R domain exist is supported by experiments locating additional phosphorylated serines in the R domain (Seibert et al., 1995).

CFTR chloride channels require PKA for initiation of channel activity and ATP for channel opening. Structural characterization of the R domain suggests that initiation of CFTR chloride channel activity by PKA phosphorylation involves conformational changes in the R domain wrought by the additional negative charge of the phosphate (Rich et al., 1993)(Dulhanty and Riordan, 1994). This change exposes the pathway through which ions traverse the membrane. Although CFTR requires PKA to prime the channel, PKA is not required for continued channel activity. The CFTR channel can remain open, in the absence of specific phosphatases, if PKA is removed. However, the

presence of specific phosphatases can result in dephosphorylation of the R domain and subsequent rundown of channel activity, which is restorable by addition of the catalytic subunit of PKA and ATP (Tabcharani et al., 1991).

1-3.1.3. Role of the Nucleotide Binding Domains

Existence of the two nucleotide binding domains is a feature unique to CFTR among ion channels, but characteristic of the ABC family of transporters. When NBD's exist in other proteins, they often serve to hydrolyze ATP as a source of energy for substrate transport. Understanding of CFTR as a chloride channel that passively transports chloride down an electrochemical gradient implies that ATP is not directly used as energy currency to move chloride. However, ATP and ATP derivatives do bind to recombinant peptides corresponding to nucleotide binding domains *in vitro* (Thomas et al., 1991). Moreover, mutations in one or both of these domains, particularly in NBD1, accounts for a disproportionate number of mutations responsible for CF, implying that whatever reaction occurs there is required for normal function (Cutting et al., 1990)(Kerem et al., 1990). Thus, the question remains whether hydrolysis occurs after binding, and, if so, whether it serves a regulatory or direct channel gating function.

Several lines of evidence bolster the argument that hydrolysis is an essential component of CFTR function. Although the channel can remain open in the absence of PKA, the channel closes if ATP is removed after PKA phosphorylation, manifest as observed rundown of activity after channel excision. Resupply of ATP, as well as other hydrolyzable nucleotide triphosphates (ATP>GTP>ITP=UTP>CTP; 8-azido-ATP), to the cytosolic surface of CFTR results in reopening of the channel (Anderson et al.,

1991a)(Travis et al., 1993). Nonhydrolyzable ATP analogs, such as compounds with modified polyphosphate moieties (ADP, AMP-PNP), and Mg^{2+}-free ATP are not effective in opening the channel. ATPγS , which is an analog of ATP with a slow rate of hydrolysis, substitutes for ATP in PKA phosphorylation, but does not successfully open the channel (Anderson et al., 1991a)(Nagel et al., 1992). In addition, the open probability (P_o) of the channel increases directly as a saturable function of ATP concentration (Venglarik et al., 1994).

While ATP is required for continued channel activity, ADP inhibits ATP-dependent CFTR channel gating. In the presence of ATP, increasing concentrations of ADP progressively inhibit current in a pattern consistent with competitive inhibition. Mutation of three sites in the second nucleotide binding domain (NBD2) abolishes this inhibitory effect, suggesting that the inhibitory effect resides with NBD2 (Anderson and Welsh, 1992). Inability of ADP to open the channel (Anderson et al., 1991a) and simultaneous competition with ATP for binding at the NBD, summarily prevents channel opening when ADP is present (Schultz et al., 1995). Understanding this relationship between the ratio of ATP and ADP leads to an explanation of how the metabolic state of the cell might determine certain cell activities. In this scheme, CFTR is able to sense the cell's energy level and modulate chloride secretion accordingly.

Interestingly, function of the two NBD's is not equivalent. Mutations in two conserved regions thought to represent sites for ATP binding found in both NBD1 and NBD2, the Walker A and Walker B consensus sequences, produce strikingly different results. Mutation of lysine of Walker A results in a decrease in sensitivity to channel

activation, while the opposite is true of mutation of the same lysine in Walker A of NBD2 (Smit et al., 1993). This recent work supports previous studies of NBD mutations proposing two ATP binding sites with differential functions (Anderson and Welsh, 1992). Inorganic phosphate analogs, including VO_4 and BeF_3, inhibit enzymes that catalyze phosphoryl transfer reactions (e.g. ATPases). When added to cell patches in which channels have been opened by addition of ATP, the channels become stabilized, or "locked," in the open conformation (Baukrowitz et al., 1994). Furthermore, AMP-PNP, which is a nonhydrolyzable analog that binds only at NBD2, could not open the channel, but did prolong P_o of channels already activated by ATP (Hwang et al., 1994). This data supports models in which hydrolysis at one or both NBD's contributes an essential component to channel gating. In these schemes, the two NBDs appear to function differently, with binding and hydrolysis of ATP at NBD1 effecting channel opening, and similar action at NBD2 resulting in channel closure (Davis et al., 1996).

Despite this growing collection of data which seems to support the role of ATP hydrolysis, controversy persists. While the NBD's can have a regulatory function via nucleotide binding without hydrolysis (i.e. via ADP competitive inhibition), the question remains whether a regulatory role exists that requires nucleotide hydrolysis. Studies of epithelia in which the basolateral membrane was permeabilized with α-toxin produced data supporting the ability of non-hydrolyzable ATP analogues to augment apical chloride conductance. In these experiments involving human sweat ducts (Bell and Quinton, 1993) and T84 cells (Quinton and Reddy, 1992), nonhydrolyzable ATP analogues activated previously phosphorylated CFTR channels. This appears to

contradict the single-channel studies described previously. Explanations abound for this seeming incongruity. Research indicates that certain ATP analogues react with some, but not all, ATPases (Moos et al., 1960). Thus, in order to discuss whether hydrolysis occurs at an NBD, one must first prove that the ATP analogue in question is in fact interacting with the protein. For example, AMP and non-hydrolyzable analogues (AMP-PCP, AMP-PNP, AMP-PCP, ATPγS) have polyphosphate moieties significantly different from ATP, and may simply not interact with the hydrolytic enzyme portion of CFTR, or may require other conditions, such as a highly phosphorylated state, to bind adequately (Schultz et al., 1995). A second line of reasoning relies on differences in experimental conditions to explain inconsistent outcomes. For example, experiments conducted at 25°C in the continued presence of PKA produced a P_o greater than that seen in experiments in which AMP-PNP was added. This indicates that the nonphysiologic temperature induces a different kinetic state, independent of the addition of the nonhydrolyzable ATP analogue (Schultz et al., 1995).

1-3.1.4. Role of Other Kinases

The role of protein kinase C (PKC) in activation of the CFTR chloride channel is less clearly defined. Recent work has identified both calcium-dependent and calcium-independent isoforms of PKC-activated CFTR chloride channels. In the calcium-independent model, PKC directly phosphorylates CFTR (Berger et al., 1993). However, conflicting studies report that while activation of PKC causes a modest increase in the magnitude of CFTR chloride currents, it more significantly confers a marked reduction in

stability of CFTR chloride currents in pancreatic duct cells (Winpenny et al., 1995). Even when PKC functions in CFTR activation, it alone stimulates only approximately 10% of the current stimulated by PKA (Tabcharani et al., 1991)(Berger et al., 1993).

1-3.2. Outwardly Rectifying Chloride Channel

Prior to identification of CFTR, the chloride channel responsible for the CF defect was thought to be an outwardly rectifying, 30-50 pS channel, called the outwardly rectifying chloride channel (ORCC). Like other chloride channels, the ORCC has distinct functional and electrophysiologic properties that distinguish this channel. The channel has an ion selectivity sequence of iodide>bromide>chloride, demonstrates voltage dependence, and is sensitive to inhibition by 4,4' diisothiocyanostilbene-2,2' disulfonic acid (DIDS). Thus, the biophysical properties differ completely from those now known to describe the small conductance CFTR chloride channel (Table 1).

However, early research on the molecular basis for the CF defect described aspects of ORCC functioning that appeared to coincide with expectations for the CF gene product. Data indicated that the ORCC was activated by cAMP agonists in cells without the CF defect (Frizzell et al., 1986; Berger and Welsh, 1991) and, inconsistently, by the catalytic subunit of PKA and PKC (Li et al., 1988)(de Jonge et al., 1989)(Hwang et al., 1989)(Jetten et al., 1989). In contrast, activation of ORCC by cAMP and PKA was defective in tissue from patients with CF (Chen et al., 1989)(Hwang et al., 1989)(Jetten et al., 1989). Of note, even in CF-affected cells, the channel could be activated with trypsin, strong depolarization, and excision at 37°C (Schoumacher et al., 1987)(Hwang et al.,

Table 1

Properties of Apical Chloride Channels

Biophysical Properties	CFTR	ORCC
Conductance	8-10 pS	20-30 pS
I/V Curve	Linear	Outward rectifying
Inhibitors	DPC Glibenclamide	DIDS
Activation	PKA ATP (intracellular)	Voltage ATP (extracellular)
Ion Selectivity	$Br^- > Cl^- > I^-$	$I^- > Br^- > Cl^-$

Definition of abbreviations: CFTR =cystic fibrosis transmembrane conductance regulator; ORCC=outwardly rectifying chloride channels; I/V curve=current voltage relationship; DPC=diphenylamine-2-carboxylic acid; DIDS=4,4' diisothiocyanostilbene-2,2' disulfonic acid; ATP=adenosine triphosphate

1989). Appearance of channel activity only under these conditions implied that ORCC was present in CF patients, but improperly regulated, and that dysfunctional regulation of this channel represented the primary defect in CF. Subsequent identification and expression of CFTR lead to research that illustrated singular properties of CFTR which were different from those expected for ORCC. Expression of CFTR cDNA in various systems did not produce a channel with biophysical characteristics consistent with those observed for ORCC (Bear et al., 1992). Furthermore, the presence of currents resembling ORCC in 'knockout' mice (genotype CFTR(-/-)) disclosed additional evidence that the two channels were distinct, and that lack of ORCC was not the primary defect in CF (Gabriel et al., 1993).

Ensuing work has substantiated that ORCC is a distinct chloride channel and a unique protein, but is regulated by CFTR. The CFTR protein is required for PKA activation of ORCC (Egan et al., 1992)(Gabriel et al., 1993)(Jovov et al., 1995). Expression of recombinant CFTR in CF-affected cell lines results not only in restoration of chloride channel activity, but also reestablishes PKA activation of ORCC (Egan et al., 1992) (Gabriel et al., 1993) (Schwiebert et al, 1994). Exactly how CFTR regulation of ORCC transpires remains unclear. One model postulates that PKA-phosphorylated CFTR conducts ATP out of the cell. On the extracellular surface, ATP acts as an autocrine stimulator of ORCC through P_{2u} purinergic receptors, which then act through direct coupling to ORCC or by activation of a G-protein associated cascade (Schwiebert et al., 1995a). Interestingly, ATP had no effect on ORCC activity without CFTR membrane incorporation, or without PKA and ATP presence on the cytosolic side of the channel (Ismailov et al., 1996). Supporting the role of extracellular ATP in activation of

ORCC is the adverse effect that ATP scavengers such as hexokinase display on ORCC chloride current. In these experiments, hexokinase converted ORCC from a PKA-sensitive mode to a PKA-insensitive mode, in the continued presence of wild-type CFTR (Jovov et al., 1995)(Schwiebert et al., 1995a).

Despite these efforts to understand how CFTR may regulate other channels, it is increasingly clear that cross regulation between channels and cellular proteins is exceedingly complex. Current investigations link both cAMP-regulated chloride channels, ORCC and CFTR, as a complex jointly regulated by a G protein, $G_{\alpha i\text{-}2}$. Data implies that $G_{\alpha i\text{-}2}$ specifically inhibits ORCC, perhaps in concert with CFTR, creating a system of negative feedback inhibition of cAMP-activated chloride current. In this study of a normal human airway epithelial cell line (9HTEo$^-$) expressing both CFTR and ORCC, stimulation of the G protein with GTP and $GTP_{\gamma}S$ decreased the open probability (P_o) of ORCC, while inhibition of the G protein by GDP_BS increased P_o. Addition of pertussis toxin, which uncouples portions of the heterotrimeric G protein from the receptor, also increased P_o and restored cAMP-activated chloride current in airway cells from CF patients (Schwiebert et al., 1995b)(Ismailov et al., 1996). Of note, uncoupling of the G protein from ORCC altered the rectification characteristics of ORCC and restored PKA sensitivity in the absence of CFTR, suggesting that both rectification and PKA sensitivity is influenced by the G protein (Ismailov et al., 1996). Additional results support the theory that while CFTR chloride channel conductance may be altered by mediating vesicle trafficking and delivery of chloride channels to the membrane, ORCC function may receive direct modulation (Schwiebert et al., 1995a).

1-3.3. Calcium Activated Chloride Channels

The calcium-activated chloride channel demonstrates that epithelial chloride secretion is regulated by mechanisms involving second messengers other than cAMP. An increase in intracellular calcium concentration stimulated transepithelial chloride movement in airway epithelia as well as an increase in opening of basolateral potassium channels (Willumsen and Boucher, 1989). Initial speculation existed as to whether the calcium-activated pathway stimulated a distinct channel, or whether the different second messengers each activated the same apical chloride channel. The possibility that the pathways culminated in a single final channel was supported by the finding that, like cAMP-stimulated ion flow, calcium-stimulated chloride conductance is also defective in cystic fibrosis affected intestine (Anderson and Welsh, 1991). However, subsequent studies indicated that in both CF affected and normal airway epithelia, addition of calcium ionophore A23187 or prostaglandin F_{2a} effected an increase in intracellular calcium which brought about an increase in chloride ion movement, without changing intracellular cAMP concentration (Hwang et al., 1989)(Anderson and Welsh, 1991). Likewise, cAMP- but not Ca^{2+}-regulated chloride conductance is defective in the CF-affected mouse oviduct (Leung et al., 1995). In contrast, colonic cells lines (T84, Caco-2, HT-29cl9a) have shown the absence of apical calcium-activated chloride current. The presence of chloride conductance in response to an increase in intracellular calcium in intestinal cells is most likely due to calcium activation of basolateral potassium channels. The resulting hyperpolarization increases the driving force for chloride movement across the apical membrane (Anderson et al., 1992).

Multiple lines of evidence support the conclusion that the Ca^{2+}-dependent and cAMP-dependent pathways operate finally on distinct chloride channels. The observation that intestinal cells appear to have a second messenger-activated apical chloride channel responsive to cAMP but not to calcium suggested that the two pathways were distinct. Stronger distinction is drawn by examining the biophysical characteristics between the two channels. Ca^{2+}-activated channels are characterized in T84 colonic tumor cells by having a linear I-V relation and time-dependent relaxations of chloride current at both depolarizing and hyperpolarizing voltages (Cliff and Frizzell, 1990). Anion permselectivity has the sequence iodide>bromide>chloride (Cliff and Frizzell, 1990). Addition of DIDS to the basolateral aspect of cultured cells blocks Ca^{2+}-activated chloride current, but has no effect on CFTR chloride channels (Anderson and Welsh, 1991). Notably, an increase in both cAMP and Ca^{2+} results in an additive increase in chloride secretion from the apical membrane (Anderson and Welsh, 1991). The combination of this data indicates that the Ca^{2+}-dependent pathway may modulate chloride secretion through a different channel than the cAMP-dependent pathway.

1-4. Inflammatory Mediators

1-4.1. Products of Arachidonic Acid

Arachidonic acid is released from cell membranes in response to inflammatory stimuli and is metabolized to a variety of substances involved in the inflammatory process, including the products of cyclooxygenase metabolism (prostaglandins, thromboxanes, and prostacyclin) and the products of lipooxygenase metabolism (leukotrienes), collectively referred to as eicosanoids. These substances are intricately

involved in the pathogenesis and promulgation of a broad spectrum of inflammatory disorders, extending from diseases of the respiratory tract, such as asthma and cystic fibrosis, to rheumatic diseases, such as arthritis and psoriasis, to resolution of myocardial infarction (Holtzman, 1991)(Sigal, 1991).

Arachidonic acid is a 20-carbon fatty acid that is a component of the phospholipid moiety of cell membranes. When a cell is stimulated phospholipases release arachidonic acid, which enters one of several metabolic cascades. The mechanism by which phospholipases are activated to release arachidonic acid is thought to involve G proteins linked to a calcium dependent second messenger system (Sigal, 1991). After release from the cell membrane, arachidonic acid is oxidized by either cyclooxygenase or lipoxygenase. Each of these enzymes catalyzes stereospecific insertion of one or two molecules of oxygen into arachidonic acid.

1-4.1.1. The Cyclooxygenase Pathway

If the cyclooxygenase pathway predominates, products include prostaglandin (PG) D_2, PGE_2, PGF_{2a}, thromboxane $A_{2,}$ and prostacyclin. The enzyme catalyzes the attachment of molecular oxygen at the eleventh carbon of arachidonic acid, with subsequent rearrangement to a cyclic endoperoxide with bridging of carbons 9 and 11. Introduction by cyclooxygenase of a second molecule of oxygen yields PGG_2, while a hydroperoxidase function of cyclooxygenase performed on PGG_2 produces PGH_2. Nonsteroidal anti-inflammatory drugs, in particular aspirin via acetylation, inhibit the cyclooxgenase activity but not the hydroperoxidase activity of the enzyme. Additional

product-specific enzymes convert PGH_2 to PGD_2, PGE_2, and PGF_{2a} (Sigal, 1991). The subscript in each prostaglandin denotes the two double bonds in the final structure. In platelets, the predominant product of arachidonic acid metabolism is thromboxane A_2 which is formed from PGH_2. Prostacyclin is also formed from PGH_2, but is produced by vascular epithelial cells.

The biologic actions of the products of cyclooxygenase activity are diverse. Thromboxane A_2, produced in platelets, has strong vasoconstricting, bronchoconstricting, and platelet aggregating effects. In contrast, prostacyclin, produced by vascular endothelial cells and both vascular and non-vascular smooth muscle cells, is a potent inhibitor of platelet aggregation and a vasodilator. Prostaglandin D_2 is the principal prostaglandin formed by mast cells upon antigen stimulation through binding to IgE receptors (Roberts et al., 1979)(Lewis et al., 1982); it acts like histamine, causing strong bronchoconstriction (Sigal, 1991). In addition, PGD isomerase is found in high concentrations in the central nervous system, suggesting that PGD_2 may have neuromodulatory effects. Prostaglandin D_2 also inhibits platelet aggregation, inreases platelet cAMP concentration, and causes peripheral vasodilation, pulmonary vasoconstriction, bronchoconstriction, and airway hyperreactivity (Holtzman, 1991). Like PGD_2, PGF_{2a} has vasoconstricting and bronchoconstricting effects, but is produced by airway epithelial cells. In contrast, PGE_2 has distinct vasodilatory and bronchodilatory actions in human tracheal epithelium. This occurs by a direct effect on smooth muscle and by an inhibitory effect on acetylcholine release. Both PGE_2 and

PGF_{2a} are present in response to inflammatory stimuli and increase chloride secretion in human airway epithelial cells (Eling et al., 1986) (Widdicombe et al., 1989). Prostaglandin E_2 has been shown to inhibit gastric acid secretion and is cytoprotective, enabling the healing of ulcers and preventing further formation.

1-4.1.2. The Lipoxygenase Pathway

Lipoxygenases are a group of iron-containing dioxygenases that catalyze insertion of one molecule of oxygen into fatty acids with a 1,4-cis,cis-pentadiene structure after initial stereospecific removal of a hydrogen atom (Holtzman, 1991). Lipoxygenases are characterized as 5-, 12-, or 15-lipoxygenase to describe the point of oxygen insertion. The first product of each enzyme is therefore 5S-, 12S-, or 15S-hydroperoxyeicosatetraenoic acid (HPETE), each of which can be further metabolized to leukotrienes, aldehydes, epoxyhydroxy acids, and glutathione adducts (Holtzman, 1991).

Unlike other enzymes in this pathway, 5-lipoxygenase is specifically stimulated by calcium and has a strict substrate specificity, requiring double bonds at carbons 5, 8, 11, and 14 for optimal leukotriene formation (Holtzman, 1991). The enzyme 5-lipoxygenase converts arachidonic acid to 5-HPETE and, subsequently, 5-HPETE to leukotriene A_4 (LTA_4). Leukotriene A_4 can either be hydrolyzed to LTB_4 or converted to LTC_4 by addition of glutathione. Additionally, catalytic removal of glutamic acid by transpeptidase produces LTD_4; removal of a glycine from LTD_4 results in LTE_4. The rate-limiting steps in leukotriene biosynthesis reside in LTA hydrolase and LTC synthase (Holtzman, 1991).

The 12-lipoxygenase enzyme was the first in this pathway to be described in humans, initially in platelets and later in leukocytes (Holtzman, 1991). The first product of this branch of the pathway, 12-HPETE, is rapidly reduced to the alcohol, 12-HETE, in most cells, and is the primary biologically active product. Both 12-HPETE and 12-HETE act as specific inflammatory mediators, notably inducing secretion of neutrophil granules and augmenting IgE-mediated mast cell release (Holtzman, 1991).

Metabolism of 15-HPETE resembles that of 12-HPETE, resulting in production of 15-HETE. Oxygenation and reduction of 15-HPETE or 15-HETE by 5-lipoxygenase yields dihydroxy- or trihydroxy-acids called lipoxin A or lipoxin B (Holtzman, 1991). The 15-lipoxygenase pathway appears to predominate in human lung tissue in asthmatics (Samuelsson et al., 1987).

1-4.2. Neuropeptides

Neuropeptides, including vasoactive intestinal peptide (VIP), Substance P, and the tachykinins (neurokinin A and neurokinin B), are a diverse group of peptides released from nerve endings of the autonomic and non-autonomic nervous systems. In addition to the cholinergic nerves of the parasympathetic system and the adrenergic nerves of the sympathetic system, nonadrenergic noncholinergic (NANC) nerves are prominent in the gut and the respiratory system, and exert a broad spectrum of physiological effects expressed through release of neuropeptides. The influence of neuropeptides on bronchial and vascular smooth muscle, ion movement, and leukocyte proliferation and degranulation render them intrinsic mediators of the inflammatory response.

Physiological response to neuropeptides depends upon localization of receptors and upon second messengers in target cells.

First described in the gut, NANC networks are not responsive to adrenergic or cholinergic agonists or antagonists (Richardson, 1979)(Barnes, 1984)(Barnes, 1986). NANC nerves often have opposing actions, with mechanisms exhibiting both vasoconstriction and vasodilation, bronchoconstriction and bronchodilation; this suggests that several different neurotransmitters are involved at each the synapse. Inhibitory NANC (i-NANC) nerves relax airway smooth muscle. In humans, the NANC system is the only direct bronchodilating effect because there is no sympathetic innervation of human airways (Barnes et al., 1991). Neurotransmitters involved in this bronchodilating property appear to include VIP and nitric oxide in some experimental models, although in humans the predominant i-NANC transmitter appears to be nitric oxide (Barnes et al., 1991). Excitatory NANC (e-NANC) elements cause bronchoconstriction that is not blocked by atropine (Lundberg et al., 1987). This non-parasympathetic bronchoconstriction is thought to be mediated by tachykinins released retrogradely from sensory nerves (Barnes et al., 1991).

Contrary to the original conception, the NANC system is not an anatomically distinct nervous system but, rather, appears to "fine-tune" the effects of the autonomic nervous system. Neurotransmitter components of the NANC system are released from autonomic nerves. For example, i-NANC bronchodilation in the human airway may be mediated by release of VIP and NO from cholinergic nerves. Vasoconstrictor effects result from release of neuropeptide Y from adrenergic nerves. Excitatory NANC

bronchoconstriction results from release of tachykinins from sensory nerve endings (Barnes et al., 1991).

1-4.2.1. *Vasoactive Intestinal Peptide*

VIP is a 28 amino acid peptide, and is one of the most abundant neuropeptides in the human lung (Ghatei et al., 1982). VIP has been localized by immunoreactive analysis (VIP-IR) to airways and pulmonary vessels; it has been found in nerves and ganglia in the posterior trachea and intrapulmonary bronchi, becoming less prominent as the airways become smaller (Lundberg et al., 1984)(Uddman and Sundler, 1987). Few VIP-ergic fibers are found peripherally in the smaller bronchioles. As discussed above, VIP is usually colocalized in parasympathetic ganglia; however, isolated ganglion cells have also been demonstrated, which usually perform like cholinergic nerves (Barnes et al., 1991). The pattern of distribution largely parallels that of parasympathetic nerves, which is consistent with the colocalization of VIP and acetylcholine (Laitinen et al., 1985). Consistent with observation of correlated function, VIP-ergic neurons are found within bronchial and vascular wall smooth muscle and surrounding submucosal and serous glands (Barnes et al., 1991). Although receptors for VIP have been discovered in airway epithelium, no VIP-ergic fibers have been (Said, 1991).

Like B-adrenergic agonists, VIP effects proceed through activation of a G protein and subsequent second messenger cascade mediated specifically by adenylyl cyclase. Cellular response to binding of VIP at membrane receptors involves activation of adenylyl cyclase and subsequent increase in intracellular cyclic AMP (cAMP). This

knowledge has enabled identification of specific high affinity receptors by immunocytochemical detection of increases in cAMP (Lazarus et al., 1986). Localization of VIP receptors has also been mapped using autoradiography (Carstairs and Barnes, 1986). Results of these investigations demonstrate a high density of VIP binding in pulmonary vascular smooth muscle, in smooth muscle of large airways, surrounding submucosal serous and mucous glands (of the ferret), and in the ciliated and basal cells of canine tracheal epithelium (Lazarus et al., 1986)(Leys et al., 1986).

VIP has also been found in leukocytes, including neutrophils, eosinophils, mononuclear cells, and pulmonary mast cells (O'Dorisio et al., 1980)(Lygren et al., 1984)(Aliakbari et al., 1987). In addition, VIP receptors are present on the surface of cells that constitute the inflammatory response, including murine and human T-lymphocytes (Ottaway and Greenberg, 1984), MOLT 4b lymphoblasts (Beed et al., 1983), human blood monocytes (Guerrero et al., 1981), and rat alveolar macrophages. The role of VIP in relation to inflammatory cells has not been completely elucidated, but VIP has been shown to inhibit release of histamine from pulmonary mast cells (Undem et al., 1983) and may have other characteristic inhibitory or stimulatory features that modulate the inflammatory response in the respiratory tract.

As stated, VIP *in vitro* is the primary source of relaxation of airway smooth muscle; adrenergic antagonists do not inhibit this function. The rich innervation of the respiratory tract with VIP-ergic fibers and the consequent bronchodilating effect observed with stimulation suggests that VIP may be an essential counterforce to the bronchoconstricting effect of parasympathetic stimulation (Barnes et al., 1991). Bronchodilation is more prominent in large airways than in smaller bronchioles, which is

consistent with the predominant VIP innervation noted in bronchi compared with the paucity of VIP-ergic fibers found in bronchioles (Carstairs and Barnes, 1986). Therefore, functional bronchodilation by VIP may lie more significantly in large airways than in progressively peripheral airways.

VIP has similar properties in systemic and pulmonary vascular smooth muscle. *In vitro*, VIP has potent relaxant effects on pulmonary vessels, independent of endothelial cells, which suggests that VIP acts directly on vascular smooth muscle rather than through an intermediate relaxant factor such as nitric oxide (Greenberg et al., 1987). The substantially greater number of VIP receptors on pulmonary vascular smooth muscle in comparison to bronchial smooth muscle indicates why VIP is approximately ten-fold more potent as a vasodilator than as a bronchodilator (Barnes et al., 1991). The consequence of this receptor distribution may be to improve delivery of blood to constricted airways during bronchoconstrictive episodes such as asthma (Barnes et al., 1991).

The juxtaposition of VIP-IR nerves with submucosal serous and mucous glands results in potent mucus secretion. ^{35}S-labeled glycoprotein secretion of mucus in ferret airway *in vitro* is significantly greater than mucus secretion stimulated by isoproterenol (Peatfield et al., 1983). The mechanism of signal transduction again relies on G-protein linked increase in cAMP.

In dog tracheal epithelium, VIP has been shown to be a potent stimulator of chloride ion transport and therefore water secretion (Nathanson et al., 1983). The effect of increased chloride transport across the apical membrane of luminal epithelial cells is an increase in luminal fluid, with implications for effectiveness of mucociliary clearance.

The presence of significant numbers of VIP receptors in human airway epithelial cells suggests that VIP may be an important modulator of this clearance system.

1-5. Inflammation in Airway Disease

Evidence suggests that inflammation plays a dominant role in the propagation of airway disease, particularly in asthma and cystic fibrosis. In each case, although the path of tissue destruction differs, the inflammatory mediators described above are involved. Increases in production of mediators result in a concomitant increase in their modulating effects on bronchoconstriction, immune function, microvascular permeability, mucus secretion, and airway fluid production. Although the effects of the leukotrienes, prostaglandins, neurokinins, and neuropeptides on bronchial and vascular smooth muscle, submucosal glands, and epithelial cells have been documented, the precise mechanisms of action of each of these mediators remain unclear.

1-5.1. Asthma

The clinical picture of asthma derives from the pathogical triad of exaggerated bronchoconstriction, smooth muscle hypertrophy, and inflammation of the small airways. A variety of exogenous factors have been identified as triggers of acute asthma attacks: allergens, exercise, cold air, upper respiratory tract infections, environmental pollutants, and, in some patients, aspirin. A portion of patients will be identified as having allergic asthma. These cases are associated with a personal or family history of allergic diseases, and often have increased levels of IgE in the serum. A large proportion of patients, however, will present with no family or personal allergy history and will have negative

skin and serum tests. They are identified as having idiosyncratic asthma (McFadden, Jr. 1994). In either case, a tendency toward hyperreactivity in the tracheobronchial tree is a commonality, manifest as unstable lung function due, most likely, to inflammatory mediators. The degree of inflammation apparent in bronchoalveolar washings parallels the clinical severity of asthma (Barnes, 1991). Pathophysiologic evidence of asthma is a reduction in the diameter of the airway lumen, brought about by a combination of bronchoconstriction, vascular congestion, and thick, tenacious mucus secretions (McFadden, Jr. 1994). These manifestations are the result of accumulation of mast cells, an increase in the number of circulating CD4 T lymphocytes, and an infiltration of eosinophils with an increased propensity to degranulate (Barnes, 1991).

Inflammatory mediators are prominent components of asthma. Although the role of cyclooxygenase products is unclear, the leukotrienes are likely contributors. Leukotrienes are potent bronchoconstrictors and increase microvascular leakage. LTD_4 receptor antagonists, MK571 and K1204 219, have significant inhibitory effects on exercise or allergen mediated bronchoconstriction (Lewis et al., 1990).

The NANC system in the lungs, of which VIP is a primary neurotransmitter, is the principal determinant of airway and pulmonary vessel relaxation, and a fundamental contributor to the secretion of bronchial fluid and mucus. Mast cell trypsin degrades VIP, and, if released from mast cells in asthmatic patients, could contribute to neurotransmitter depletion (Barnes, 1991), thus tilting the balance between NANC relaxation and cholinergic constriction. Data from immunoreactive staining of lung tissue obtained from patients with and without asthma demonstrated loss of VIP from pulmonary nerve fibers in those patients with fatal asthma, regardless of the severity of inflammation

(Ollerenshaw et al., 1989). This study did not answer the question of whether this loss was a cause or a result of asthma. Nor could the study conclude whether absent VIP was secondary to loss of nerves containing VIP, decreased production of VIP by nerves, or increased breakdown of VIP by resident airway enzymes. This data supported a hypothesis proposed a decade previously that depletion of VIP in asthmatic patients might be the cause of characteristic bronchial hyperresponsivity and inflammation (Richardson, 1979)(Matsuzaki et al., 1980).

Work with lung tissue from asthmatic patients also demonstrated an increase in the density of Substance P-immunoreactive nerves in asthmatic lungs, indicating that hypersensitivity could be due to a relative imbalance between the relaxant properties of VIP and the constrictor properties of Substance P (Ollerenshaw et al., 1989). Stimulation of sensory nerves on airway mucosa can increase vascular permeability, leading to extravasation of plasma proteins into the airway lumen, vasodilation, neutrophil adherence, mucus secretion, and bronchoconstriction. Vessels in the pulmonary system are exquisitely sensitive to such neurogenic stimulus. Moreover, this effect may be potentiated by infection (McDonald, 1992).

The reduced cross sectional area of the airway lumen in asthma is not due solely to bronchoconstriction. Rather, the bronchoconstrictor response is potentiated by the degree of amplification of luminal fluid, and its composition, during the inflammatory response of asthma. Neurogenic inflammation (via leukotrienes, Substance P, and neurokinin A) is associated with leakage from venules lying beneath the epithelium; the resultant exudative fluid is rich in protein and inflammatory mediators, which act on the airway epithelium to permit leakage into the airway lumen (Yager et al., 1991). Once

across the epithelial barrier, fluid fills the interstices between epithelial folds. The fluid filling these spaces directly reduces luminal cross sectional area. In addition, if surfactant is not present in these larger airways, the increase in radius of curvature between points of epithelium may promote airway closure secondary to high surface tension (Yager et al., 1991). The net sum is the severe increase in airway resistance observed in asthma.

A possible therapeutic role for VIP in asthma was investigated in human asthmatics even prior to its known role as an anti-inflammatory agent. In normal patients, intravenous infusion of VIP had no effect on airway conductance (Palmer et al., 1986). In contrast, VIP administration resulted in significant bronchodilation, measured by improved FEV_1, and attenuated histamine-induced bronchoconstriction, in seven atopic asthmatics (Morice et al., 1983). Results with inhaled aerosolized VIP have varied: in six atopic patients, inhaled VIP reduced airway reactivity to histamine, but did not reduce airway resistance independently (Barnes, 1984). In another experiment involving patients with severe asthma, inhaled VIP did impart improvement in airway conductance, but not to the same degree as inhaled B-adrenergic agonists (Said, 1988). It is possible that the lack of bronchodilatory response seen with inhaled VIP may be secondary to poor transit of the peptide across the bronchial epithelium (Barrowcliffe et al., 1986).

Absence of VIP in the lungs of asthmatic patients could be a primary solitary defect creating a permissive environment with decreased attenuation of bronchoconstriction and increased microvascular permeability, two of the hallmarks of asthma. In addition, lack of the stimulus of VIP for ion and water transport could deplete

luminal secretions, thereby rendering airway fluid thick and immobile. The ultimate consequence of decreased VIP, whether absolute or relative to other mediators, might be a precarious imbalance in the forces acting on an asthmatic's lungs.

1-5.2. Cystic fibrosis

Cystic fibrosis, an autosomal recessive disorder, is the most common lethal genetic disease of Caucasians. The CF gene has been localized to the long arm of chromosome 7; the multitude of mutations at this region are responsible for the variety of phenotypes characteristic of this disease. The most common mutation, deletion of a phenylalanine at position 508 (ΔF508) which accounts for genotypic makeup of 70% of CF patients, results in debilitating disease of the exocrine pancreas function and lung if not aggressively treated. With the development of pancreatic enzyme replacement therapy, lung disease has become the primary source of morbidity and mortality, accounting for 90% of deaths in CF patients (Davis et al., 1996). The clinical features of CF are dominated by obstruction of the airways by a thick, tenacious mucus, with superimposed bacterial infection and colonization by *Pseudomonas aeruginosa*. Only about 0.1% of CF patients with lung disease have normal sweat chloride concentration (Davis et al., 1980). Approximately 85% of patients demonstrate pancreatic insufficiency as a result of inspissation of pancreatic ducts, with subsequent fibrosis and deterioration in exocrine function (Collins, 1992)(Sferra and Collins, 1993). Associated findings include infertility in males due to congenital bilateral absence of the vas deferens, meconium ileus, and liver disease. CF is a clinical diagnosis, consisting of sweat chloride

concentration greater than 60 mEq/ml, chronic pulmonary disease or pancreatic insufficiency, and/or history of CF in a sibling or first cousin (Quinton, 1986).

In people without CF, the CFTR protein is localized to the apical membranes of epithelial cells (Denning et al., 1992b). While ΔF508 is the most common mutation, there are at least 500 other mutations identified (Tsui, 1992), each producing phenotypes of varying severity. Variability in the site of mutation parallels the wide array of phenotypic manifestations that comprise the clinical spectrum of CF disease. Each of these groups of mutations provides a different avenue for therapeutic approaches to the disease based on modulation of the specific defect. The mechanism by which the genotypic mutation creates an inadequate protein product falls into one of four distinct categories:

Category 1 Defective Protein Production: This class of mutations includes those in which splice site abnormalities, frameshift mutations, or missense mutations produce premature stop signals (Tsui, 1992). These proteins are often unstable and, even if produced, are either degraded by the cell or have no function. Thus, these mutations result in little or no functional CFTR protein anywhere in the cell.

Category 2 Defective Protein Processing: In this group of CF mutations, CFTR is made by the cell, but is inappropriately trafficked. The ΔF508 mutation falls into this class. In sweat duct glands, antibodies directed at CFTR do not localize to the apical membrane of cells affected by this mutation (Kartner et al., 1992). Although the reason for this mislocalization is not established, errors in the secondary structure producing improperly folded protein seem likely. The abnormal structure marks the protein for degradation rather than movement to the plasma membrane. Of note, when CFTRΔF508

is grown at a lower temperature (23-30°C), some of the mutant protein does appear in its fully glycosylated form in the cell membrane (Denning et al., 1992a). Reports of whether CFTRΔF508 that is properly processed retains its ability to regulate chloride conductance vary from a form with activity reduced by one third (Denning et al., 1992b) to one with normal activity (Li et al., 1993). If CFTRΔF508 does represent a potentially normally functioning chloride channel, than efforts aimed at correcting abnormal trafficking of the mutant protein or, alternatively, of augmenting conductance of channels that do reach the membrane, could prove to be of high therapeutic yield.

Class 3 Defective Regulation: A disproportionate number of missense mutations have been reported in the nucleotide binding domains of CFTR. When mutations occur that disrupt binding and/or hydrolysis of ATP, cells produce CFTR with an absolute reduction in function or with a requirement for higher concentrations of ATP to be effective (Anderson and Welsh, 1992). Mutations have also been noted in the regulatory domain, but these appear less frequent than in the nucleotide binding domains, perhaps because there are multiple degenerate phosphorylation sites in the R domain. Any single phosphorylation site mutation does not cause lack of function (See Section 1-3.1.2.).

Class 4 Defective Conduction: The transmembrane domain is responsible for the ion specificity of CFTR. Mutations in this domain produce CFTR in which the protein is properly processed, appears in the apical membrane, and, in fact, does generate cAMP-dependent chloride currents. In all three documented mutations of this type macroscopic current properties resembled those of normal CFTR, but the single channel conductance

was reduced. The resulting total amount of current was therefore diminished (Sheppard et al., 1993).

The fundamental disorder of the mutant CF gene is the lack of production or mislocalization of a normal-functioning CFTR protein, which, as described above, regulates chloride conductance via PKA-dependent phosphorylation. Consequent derangement in chloride ion movement results in an inability to appropriately control airway intraluminal fluid, with ever-worsening disruption of mucociliary clearance. Impaired clearance permits prolonged exposure to noxious stimuli. Although direct measurements of mucociliary clearance in CF patients have yielded data revealing retarded, normal, and increased rates of clearance, patients with clinically more severe pulmonary disease usually have more substantially impaired mucociliary clearance (Sanchis et al., 1973).

Early investigations revealed a deficiency in VIP innervation of sweat glands in patients with CF (Heinz-Erian et al., 1985). Although this data was gathered prior to identification of CFTR and knowledge of the primary defect in CF, investigators recognized the increase in transepithelial potential difference in both sweat gland ducts and respiratory mucosa, and had attributed this to a decrease in epithelial chloride permeability (Quinton, 1983). Sweat glands, like respiratory epithelium, are richly innervated by the autonomic and NANC nervous systems. As discussed above, VIP is known to increase chloride conductance across canine tracheal epithelium. It remains unclear whether VIP increases chloride conductance through stimulation of CFTR chloride channels, through activation of the ORCC, or by means of the paracellular pathway. If the absence of VIP innervation of sweat glands reflects a systemic depletion was achieved. The resulting [illegible]

et al., 1993).

The mechanism of [illegible]

immobilization [illegible]

regulates [illegible]

development of [illegible]

airway [illegible]

hormonal [illegible]

anabolic [illegible]

muscle [illegible]

pulmonary [illegible]

[illegible] et al., 1993).

Early [illegible]

patients with COPD [illegible]

identification [illegible]

recognized the [illegible]

and respiratory [illegible]

permeability [illegible]

important in the [illegible]

known to increase [illegible]

[illegible]

[illegible]

[illegible]

of VIP innervation, then lack of normal stimulation of alternative chloride current routes may represent an additional defect in CF that adds to the basic defect in chloride channel operation, and, thus, contribute to desiccation of the airways. If VIP innervation of airways were intact, then understanding of the mechanism of increased chloride conductance via VIP might provide an opportunity for luminal fluid modification.

The clinical course between the initial genetic defect and the evolution of irreversibly damaged lungs is characterized by recurrent suppurative infections instigated by *Pseudomonas* colonization. Patients exhibit a profound inflammatory response which is self-propagating: neutrophil elastase, released in response to *Pseudomonas*, destroys key opsonins, such as IgG and C3bi, as well as opsonic receptors on the surface of macrophages (Davis et al., 1996). Elastase also stimulates neutrophil chemoattractants, including LTB4 and IL-8, increases mucus secretion, and causes structural airway damage (Bruce et al., 1985)(Nakamura et al., 1992). Additional data demonstrates that certain strains of *Pseudomonas* express phospholipase C, a hemolysin which directly induces an inflammatory response when injected into mice. The response is mediated by an increase in inflammatory cells, plasma protein, and the concentration of both arachidonic acid metabolites (LTB_4, LTC_4, LTD_4, LTE_4) and cyclooxygenase products (PGF_{2a}, TXB_2) (Meyers and Berk, 1990). This data, combined with previous work showing the presence of antibodies to phospholipase C in 100% of CF patients, may explain the chronic inflammation present in CF patients (Granstrom et al., 1984). The constitutive release of cytokines induced by leukocytes and *Pseudomonas* products was recently confirmed, although the heightened cytokine levels were not ascribed to the CFTR protein defect itself (Bedard et al., 1993).

It has been proposed that the fundamental interference with mucociliary clearance is the determining factor in the ultimate colonization with *Pseudomonas.* However, consistent colonization with *Pseudomonas* in CF patients amidst variability in mucociliary clearance across CF patients draws into question the causality of a reduced clearance. Moreover, while post-infection involvement of elastase and the clear response to *Pseudomonas* products may explain the chronic inflammation observed in these patients, there remains a gap in understanding why these patients are prone specifically to pseudomonal infection. Some researches speculate an increased adherence of *Pseudomonas* to CF epithelium (Zar et al., 1995). Other research suggests that the inflammatory response is itself an integral part of CF, and may predate an intensification of and predispose to infection. Bronchoalveolar lavage fluid from CF infants reveals an increase in neutrophils and IL-8, without evidence of intercurrent infection (Khan et al., 1995). Likewise, lipid mediators -- leukotrienes and prostaglandins -- have long been identified in CF sputum, yet treatment with antibiotics does not always decrease eicosanoid levels in sputum and other respiratory fluid (Zakrzewski et al., 1987).

Recent investigation suggests that airway epithelia secrete a bactericidal substance into the fluid bathing the apical surface which depends on low NaCl concentration to function properly. CF patients have a high chloride concentration on affected epithelial surfaces secondary to loss of CFTR chloride channels. This research demonstrated that typical CF pathogens were killed when added to normal epithelia. Moreover, when the NaCl concentration bathing CF epithelia was lowered from its pathologic level, *Pseudomonas* was killed (Smith et al., 1996). The authors suggest therefore that the abnormality in sodium and chloride transport not merely affects host defenses by

impairing mucociliary clearance, but more fundamentally alters the host's primary chemical defense. This line of reasoning requires a model in which respiratory epithelium is a chloride absorbing, rather than a chloride secreting epithelium, which is in conflict with the larger body of CF research. Of particular concern in evaluation of this argument is how the luminal concentration of NaCl could become so large (124-249mM), given that respiratory epithelium is not considered to be a leaky barrier. That is, even in the absence of postulated absorptive CFTR chloride channels, how could the luminal chloride concentration become hypertonic to the plasma?

While these various arguments each has appealing qualities, none fully clarifies how sodium and chloride ion channels work precisely, or how abnormalities in this function coalesce to create the clinical picture of CF. Nevertheless, all agree that there is a baseline dysregulation of chloride movement, secondary to mutation of the CFTR protein. Understanding more about the structure of this protein, the various channels it may regulate, and means to manipulate the CFTR gene product through pharmacologic modulation is essential both to resolving these conflicting data and to providing useful therapies for those afflicted with CF.

2. Statement of Purpose

The objective of this study was to employ electrophysiological techniques (cell-attached and excised inside-out patch clamping) to describe the effects of a specific inflammatory mediator, vasoactive intestinal peptide (VIP), on chloride conductance in cultured airway epithelial cells. The following questions were addressed in this study:

1) Does pretreatment of cultured airway epithelial cells with VIP result in increased chloride conductance in the cell-attached or excised inside-out patch?

2) Does VIP increase chloride conductance through a transcellular or paracellular pathway?

3) Is there a statistically significant difference in VIP-stimulated chloride conductance in airway epithelial cells from a cystic fibrosis patient (IB3-1 cell line) and in “complemented” cells (S9 cell line)?

4) Are the functional characteristics of observed channel activity consistent with previously described apical chloride channel types, specifically, the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel or the outwardly rectifying chloride channel (ORCC)?

5) What are possible cellular mechanisms of observed increases in chloride conductance after pretreatment with VIP?

6) What is the potential therapeutic value of VIP in airway disease?

3. Methods

3-1. Patch Clamp Technique

3-1.1. Theory and Configurations

Patch clamp technique was initially conceived of to permit isolation and characterization of currents flowing through single channels in the membrane of cells. Continued efforts have resulted in refined techniques and equipment that permit increasingly higher resolution of channels and advanced manipulation of the environment surrounding the cells to be studied. These improvements have made it possible to study a wide variety of cell types.

Patch clamp experiments are a specific type of voltage clamp experiment, that is, one controls the voltage and measures the transmembrane current required to maintain that voltage. Provided that no other parameters influence physiologic functioning of the cell, examining cell membranes under conditions in which the voltage is controlled allows determination of the effect of shifts in voltage and evaluation of the effect of any specific mediator if the voltage is held constant. The opening and closing, or "gating" of a channel can be quantified by measuring the time a channel conducts. Conductance, measured as change in single channel current for a small change in membrane voltage, is roughly constant for a given channel type (Corey, 1983). Units are in amperes per volt, or siemens (S).

Currents are observed and recorded by placing a glass micropipette flush against the external membrane of the cell to be studied. The current recorded is the current

flowing across the membrane encircled by the tip of the micropipette. Because the cross-sectional area at the tip of the pipette is so small, the probability that there is only one or two channels in the patch of membrane being recorded is high.

Patch clamp configurations are designated by the orientation of the membrane in relation to the micropipette, and this configuration determines whether the applied voltage is hyperpolarizing or depolarizing (Sherman-Gold, 1993). There are four basic configurations for clamping: cell-attached, inside-out, whole cell, and outside-out (Figure 3). Each has advantages and disadvantages; the type used for a given experiment is determined by the type of cell and channel being studied.

In *cell-attached* patches the membrane patch remains attached to the cell, with the pipette tip connected to the outside surface of the membrane. In this case, a positive command voltage (V_{com}) causes the transmembrane potential (V_{mem}) to become more negative, and thus is hyperpolarizing. This configuration is least disturbing to the structure and internal milieu of the cell. While this may be beneficial for obtaining the most physiologic data, it limits manipulations one may make. The inner membrane faces the cytoplasm of the cell, which one cannot control. Moreover, to measure the resting intracellular potential, one must insert a separate voltage-measuring electrode.

An *inside-out patch* is created when the pipette is retracted from the surface of the cell suddenly after a seal is achieved; the surface that was originally the inner membrane is then exposed to the bath solution, while the previously external membrane now faces the inside of the pipette. Thus the potential on the true inside surface is 0mV, the bath potential. The transmembrane voltage can be known exactly. In this case, a positive command voltage causes the transmembrane potential to become more negative, and thus

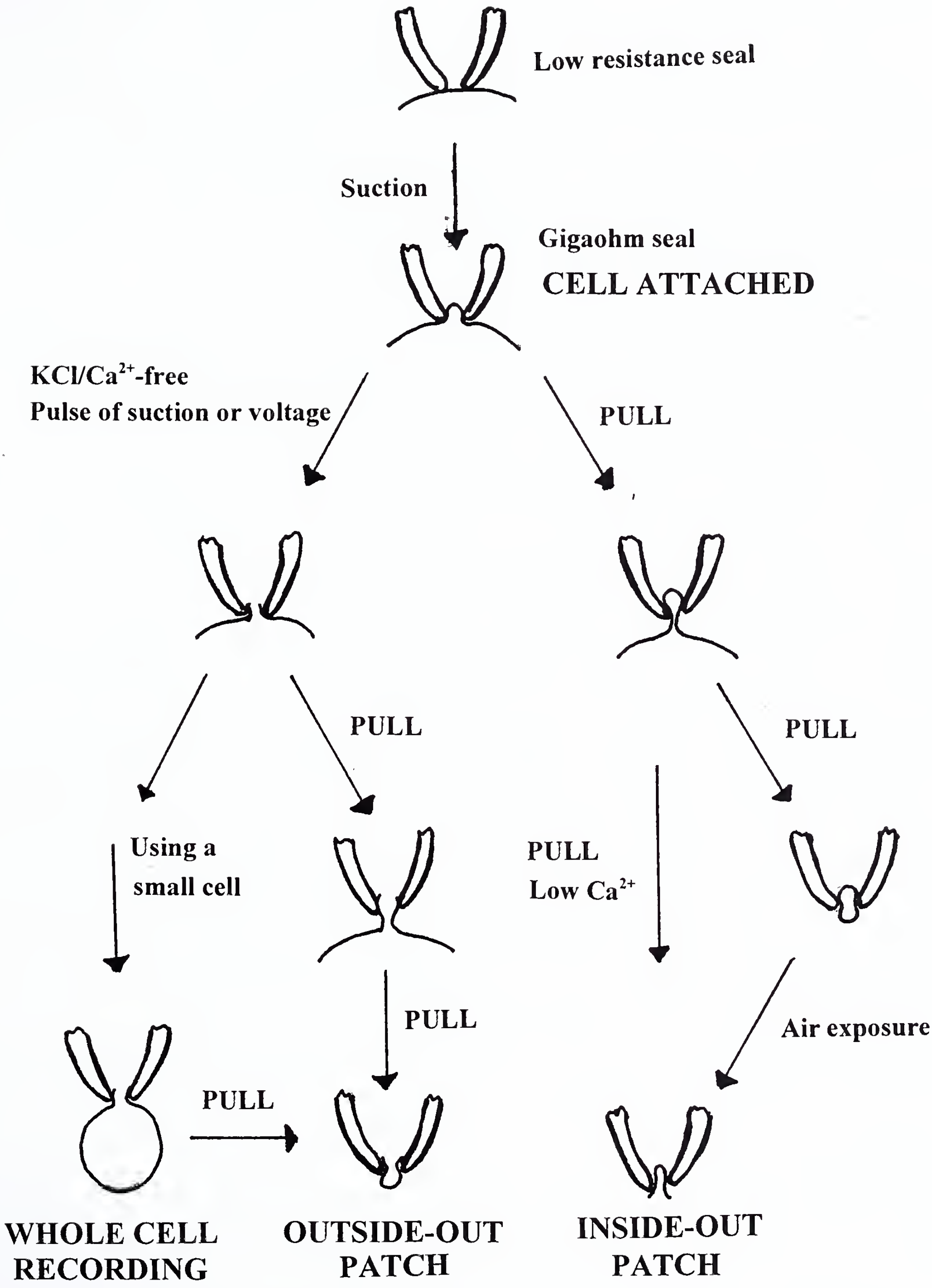


Figure 3. Four patch clamp configurations. This is a schematic illustration of four different patch clamp configurations and the manipulations that lead to them. Please see text for details. Adapted from Hamill, et al., 1981.

is hyperpolarizing. A key advantage to the inside-out patch is the unlimited access one has to the cytoplasmic surface of the membrane: during the course of an experiment one may change the concentration of any mediator or ionic consituent without disrupting the integrity of the membrane. The primary disadvantage to this configuration is that the patch has a propensity to seal over and form a vesicle, which obstructs access to the inner surface of the membrane and can alter current flow. The risk of vesicle formation can be minimized by using a bath solution with micromolar calcium concentration, which can be achieved by the addition of chelators such as EGTA (Corey, 1983). If a vesicle does form, it can sometimes be broken by briefly withdrawing the pipette from solution. Inside-out patch clamping was the sole configuration employed in these experiments.

In a *whole-cell* patch, the membrane remains attached to the cell, but a brief pulse of suction results in rupture of the membrane just inside the rim of the pipette. This configuration is similar to impaling a cell with a microelectrode, but produces good recordings on very small cells (Sakmann and Neher, 1984). In whole-cell patches, the average activity of all channels in the patch is measured, rather than that of individual channels. Thus, this method is useful when the conductance of a given channel is too small to be measured as a single event (Corey, 1983).

An *outside-out* patch is derived from a whole-cell patch by slowly withdrawing the pipette, thereby pulling a tube of membrane away from the cell until the portion of membrane pinches off. The membrane patch is detached from the cell such that the surface that was the outside of the cell remains exposed to the bath solution, and retains its original 0mV potential. As the inverse of the inside-out configuration, the advantage lies in being able to alter the ionic, hormonal, or neurotransmitter environment on the

outer surface of the membrane. Outside-out patches are also less likely to form vesicles (Corey, 1983). In an outside-out patch, a positive command voltage causes the transmembrane potential to become less negative, and, hence, is depolarizing.

3-1.2. Micropipettes

Patch clamp microelectrodes consist of an AgCl-coated Ag wire placed in a solution-filled glass micropipette, which serves as the reservoir for the conducting solution and the means of connection to the cell membrane. Early problems in patch clamping centered on obtaining and maintaining an adequate seal between the edges of the pipette and the cell. Adequacy of the seal depends on the resistance obtained. When high seal resistance is achieved, a greater fraction of the membrane current flows into the pipette rather than leaking out. If there is not a tight adherence between the membrane and the glass of the pipette, current can leak into the extracellular solution, which adversely affects recordings obtained: 1) current lost through leakage will not be measured; 2) thermal movement of charges through the conducting solution will result in increased noise in the recording (Sherman-Gold, 1993). Thus, a high resistance seal is essential to minimize undetected current and to produce low-noise recordings. Such a seal is referred to as a "gigaseal," and is defined as a sudden increase in seal resistance by as much as three orders of magnitude (Hamill et al., 1981). When a gigaseal is formed, noise due to current leakage becomes minimal; other sources of noise, including that from electronics and the micropipette glass, become the limiting factors in recording resolution. The two main criteria for achieving gigaseals are use of filtered solutions in the bath and the pipette, and use of a fresh pipette for each seal (Hamill et al., 1981).

Obtaining gigaseals consistently requires that one use clean, freshly fabricated glass micropipettes, and that they only be used once. Manufacture of pipettes entails a three step procedure: pulling of the pipette, coating of the shaft with a hydrophobic substance, and heat polishing of the tip (Hamill et al., 1981). Pipette fabrication equipment includes a Narishige (Tokyo, Japan) PP83 puller and MF-83 microforge. Glass capillary tubes that are appropriate for the production of micropipettes have walls of substantial thickness (0.2-0.3mm), which generates less electrical noise and increases bluntness at the tip, thereby reducing the possibility of cell penetration during seal formation (Sherman-Gold, 1993). Garner glass micro-hematocrit capillary tubing (Borosilicate, Kimax KG-33 and Corning 7052), with an inside diameter of 1.05mm and an outside diameter of 1.5mm, is pulled using a two step process in order to ensure a suitably blunt tip. In addition to wall thickness, three features of the glass used to fashion pipettes contribute to the quality of the recordings obtained: the temperature at which the glass softens, the electrical properties of the glass, and the major chemical constituents of the glass (Sherman-Gold, 1993). Glasses that soften at low temperatures produce a blunter tip than glasses that soften at higher temperatures. Borosilicate capillary tubing softens at 821°-830°C. The dielectric properties of a particular glass are described by its "loss factor;" glasses with the lowest loss factor exhibit the lowest noise. Finally, glass made of components with a tendency to leach into the surrounding solution can unfavorably alter the currents observed.

To minimize noise resulting from solution creeping up the outer walls of the pipettes, the shafts of the pulled pipettes are coated with a hydrophobic compound,

Sylgard (Dow Corning, Belgium). Ideally, pipettes are coated to within 100μm of the tip, and then cured by passing the tip within a heated metal coil. However, this procedure was not performed with the latter half of experiments in this study, and appeared to make no difference in the data obtained. An optimally blunt tip is obtained by firepolishing the pipette tip.

3-1.3. Channel Recording and Analysis

Data is acquired using the Axopatch 200 integrating patch clamp and the Pclamp6\TL-1 data acquisition and analysis system. Data are digitalized (Sony PCM-601 ESD1 audio processor) and stored on either Sony T120PR or Maxel T120P/I Plus professional grade VHS videotape. Channel data is viewed on an oscilloscope (Nicolet 300).

Recordings are transferred to computer (Gateway 486 computer) in 20 second sweeps in digitalized form by either an Axopatch TL-1 or a Digidata 1200 interface. Digitalized files are analyzed using Pclamp6 software (Axon Instruments, Burlingame, CA). All recordings are analyzed at a filter frequency of 300 Hz. Pclamp6 software was used to determine mean channel amplitude for each 20 second sweep. A Marquardt Least Squares fit was employed to determine mean amplitude.

Mean current for each voltage level was determined by Sigmaplot software. I-V plots were generated, and linear regression of the resulting plot yielded a line, the slope of which equals the mean current of all channels observed.

3-2. Tissue Culture

Three cell lines, each with specifically chosen and constructed properties, were used in these experiments. The IB3-1 cell line consists of clonal descendants taken from a cystic fibrosis-affected child, and thus lacks normal functioning CFTR protein. The S-9 cell line is the IB3-1 cell line transfected with wild type CFTR. The FHTE cell line is obtained from normal fetal human tracheal epithelium. Each of these cell lines were obtained from Dr. William Guggino at Johns Hopkins University. Dr. Marie E. Egan is presently culturing these cell lines at Yale University School of Medicine.

Tissue culture techniques employed consist of growing cells in T75 flasks coated with a mixture of distilled water, fibronectin (10µg)/bovine serum albumin (BSA)(100µg)/collagen (6mg/ml) and maintained in a 5% CO2/95% air incubator at 37°C. Growth media, LHC-8 supplemented with penicillin (100µg/ml), streptomycin (100µg /ml), tobramycin (80µg/ml), 5% fetal calf serum, and endothelial cell growth factor (25µg/ml), is changed three times a week. Prior to patch clamp studies, cells are passaged onto glass chips coated with fibronectin, BSA, and collagen as described above. They are maintained as described above, and are studied optimally 2 days after passage.

3-2.1. IB3-1 Cell Line

The IB3-1 cell line was created to permit detailed investigation of the specific genetic defect known to lie behind the lethal disease cystic fibrosis. Zeitlin, et al., used bronchial epithelium from a patient with CF to establish this immortalized cell line (Zeitlin et al., 1991). They infected cells with AD-12-SV40, a hybrid of adenovirus 12

(AD-12) and simian virus 40 (SV40), which had been used successfully in previous efforts to transform human epithelial cells (Rhim et al., 1985). These earlier studies with AD-12-SV40 had shown that the virus induces mRNA from the transforming region of SV40 during transformation. Moreover, it has been shown not to be tumorigenic.

Bronchial epithelial cells were gathered from the airways of a 7-year-old white male child determined to have CF by sweat chloride test and the presence of severe obstructive pulmonary disease accompanied by colonization with *Pseudomonas aeruginosa*. The primary cells (CFBE) were maintained in a mixture consisting of medium supplemented with endothelial cell growth supplement and antibiotics. The CFBE cells were infected with an AD-12-SV40 hybrid after the primary culture reached 80% confluence. Within 4 to 6 weeks, clusters of clonal growth were apparent. At passage 12, CFBE cells were diluted and plated, and, after screening and selection of only those cells that were non-producers of virus, the clone IB3-1 was cloned a second time.

Radioimmunoassay quantitation of cAMP, genetic analysis, and patch clamp analysis studies were performed to determine the characteristics of the final IB3-1 clone. Five criteria were used to judge suitability of this cell line as representative of CF epithelia:

1. The cells must consistently demonstrate abnormal chloride permeability in the apical membrane and failure of stimulation of chloride conductance by B-adrenergic agonists;

2. B-agonist stimulation should increase intracellular cAMP;

3. the secretory chloride channel should open with depolarizing voltages, but not in response to phosphorylating agents (e.g. PKA);

4. the DNA must contain the mutant CF gene;

5. long-term propagation of cells in culture must be possible without loss of phenotype.

The final IB3-1 clone satisfied all of these requirements. Of note, the patient from whom the CFBE were harvested was not homozygous for the most common CF mutation, ΔF508. These cells are genotypically ΔF508/W1282X at the CFTR locus. The W1282X mutation creates a premature stop codon, producing an unstable message and no CFTR protein. Thus, unlike previous cell lines which only conveyed information about epithelial cell characteristics in the presence of homozygous mutations, this cell line provides information regarding the effects of alternate mutations on chloride conductance.

3-2.2. S9 Cell Line

The S9 cell line is the IB3-1 cell line transfected with the wild-type CFTR gene. With this configuration, data obtained from similar experiments on the two cell lines permits correlation of the effect of experimental manipulations on the S9 cells specifically with the presence of a normal functioning CFTR-associated chloride channel. By creating the “normal” cell line from the defective one, the presence of confounding differences in other aspects of cell function is eliminated.

Flotte, et al., used an adeno-associated virus (AAV) vector to transfect the full length CFTR cDNA, and demonstrated restoration of cAMP regulation of chloride conduction ascribed to proper CFTR functioning (Flotte et al., 1993). AAV vectors consisting of a p5 transcription promoter and the AAV inverted terminal repeat (ITR) were used in the development of the IB3-1 line. However, the packaging parameters of foreign DNA limit its use when the material to be transfected is a large gene, such as CFTR. Flotte et al., recognized that the AAV ITR was able to function directly as a transcription promoter. CFTR cDNA with an amino-terminal deletion, expressed via an AAV ITR promoter, effectively complemented the CF defect in the IB3-1 cell line.

Cells were tested for functional expression of CFTR by examining the response to forskolin via chloride efflux assays. To correlate the appearance of cAMP-associated increases in chloride conductance with expression of CFTR protein, cells were stained with anti-CFTR antibodies and examined via immunofluorescence. Results demonstrated that only cells treated with AAV vectors expressing functional CFTR protein stained significantly in the immunofluorescence assay. Finally, Southern blot analysis confirmed the presence of replicating forms of AAV-CFTR vector DNA in all cells treated with vectors.

3-2.3. FHTE Cell Line

The FHTE cell line was created by Gruenert, et.al., as an object for detailed studies of the mechanisms of normal epithelial chloride ion transport (Gruenert et al., 1988). Cells were isolated from human fetal trachea. A plasmid consisting of the simian virus 40 (SV40) genome which was defective in the origin of replication (pSVori-) was

chosen because this deficiency had previously been demonstrated to enhance transformation in human fibroblasts. Although transformed cells experience a change in phenotype, epithelial cells retain important aspects of the original phenotype after transformation crisis, including responsiveness to neural agonists (e.g., B-adrenergic agonists), keratin production, and chloride ion transport.

The residual phenotypic characteristics of these cells were used to assess success in obtaining an immortalized cell line suitable for experimentation. All cell lines tested expressed immunoreactivity to keratin in both pre- and post-crisis stages. Cells were exposed to the B-adrenergic agonist, isoproterenol, to determine whether the cells retained the second messenger cascade involving cAMP. Stimulation with 10^{-5}M isoproterenol led to an accumulation of cAMP.

Of note, although the respiratory epithelium is a primary site of cystic fibrosis, studies have demonstrated that CF lung is normal *in utero*, even when disease is present in the intestine, vas deferens, pancreas, and liver (Sturgess and Imrie, 1982). This suggests that the fetal tracheal chloride channel may operate under different regulation than the adult respiratory epithelial chloride channel. The lack of disease in the fetal lung is surprising, given the plethora of CFTR activity present before birth (McCray, Jr. et al., 1992). If the fetal lung does have alternative chloride channel stimulation mechanisms, then the FHTE cell line may not be optimal for study of CFTR properties.

3-3. Protocol

Patch clamp experiments were performed using cell-attached and excised inside-out membrane patches from normal (FHTE), CF-affected (IB3-1) and CFTR-transfected (S9) cell lines. All experiments were conducted at 25-30°C. Bath solution was changed between each experiment with a 5mL flush. Of 25 experiments included in this report regarding the effect of VIP, each represents data obtained from the cell-attached and excised membrane of a distinct epithelial cell.

All cells are pretreated with 1μM VIP for ten minutes prior to seal formation. Cells are visualized with a Nikon Diaphot phase contrast microscope which is mounted on a Newport anti-vibration airtable to minimize turbulence. A fresh clean flint glass micropipette for each patch attempt is positioned by means of a Newport micromanipulator. A cell attached configuration is achieved by gently pressing the patch electrode against the cell membrane of the intact cell. Application of negative pressure generated by withdrawal of a 10mL syringe provokes development of a high resistance seal. After gigaseal formation, patches are observed in the cell-attached position. Membrane potential is varied using Pclamp software (Axon Instruments, Burlingame, CA) in 10mV steps from +80mV to -80mV, beginning at +40mV and moving sequentially outward toward +80mV. Patches are then excised by retracting the pipette briskly from the cell to obtain inside-out patches. Membrane potential is again varied in a stepwise fashion.

During recording of excised patches, each patch is observed for rundown in channel activity representative of depletion of ATP from the bath solution. If rundown is

noted, exchange of pure bath solution with bath solution containing ATP (1mM) is attempted with maintenance of the seal.

Solutions and Chemicals

Bath Solution: 150mM NaCl, 2mM$MgCl_2$, 1mM EGTA, 5mM Hepes, 0.5 mM $CaCl_2$

Pipette Solution: 150mM NaCl, 2mM $CaCl_2$, 5mM Hepes

The pH of each solution was adjusted to 7.3 with Tris base. All solutions were filtered at the time of use with a Millipore 0.22 filter.

Synthetic porcine VIP was suspended in standard bath solution to make a stock solution (1mM). The stock solution was frozen until the day of use. A 1μM solution was made by adding 40μl of stock solution to 40mL of standard bath solution. VIP was obtained from Sigma Chemical Co. (St. Louis, MO), product number V3628.

The magnesium salt of ATP was dissolved in standard bath solution to a final concentration of 1mM. The pH of the solution was adjusted to 7.4. ATP solution was kept on ice between experiments; remaining ATP solution was frozen for up to one week. ATP was obtained from Sigma Chemical Co. (St. Louis, MO), product number A-9183.

4. Results

4-1. FHTE Cell Line

Although initial experiments were conducted with the FHTE cell line, difficulties with maintaining the cells and with patching them prompted continuation of the study with the IB3-1 and S9 cell lines. FHTE cells did not consistently reach confluence in a specified time period and were prone to contamination. Cells that appeared amenable to experimentation proved to be fragile and did not seal reliably. Preliminary data from experiments conducted with FHTE is similar to data presented here for S9 cells. However, the tracings were brief and problematic to analyze due to electrical noise. Thus, FHTE data is not documented in this report. All of the analyzed data is derived from experiments involving the IB3-1 and S9 cell lines.

4-2. IB3-1 Cell Line

A gigaseal was successfully obtained and held in thirteen patches of IB3-1 cells. Each of these cells had been pretreated with 1μM VIP solution for ten minutes prior to seal formation. Patches were first observed in the cell-attached mode. Voltage potential was initially held at ±40 mV, with recording of the corresponding oscilloscope image made at 10mV increments up to ±80 mV. After channel activity, or the lack thereof, had been documented at each level, the patch was excised, and again exposed to holding potentials ranging from ±40 mV to ±80 mV. Of the thirteen patches in which a gigaseal was attained and recordings made in the cell-attached mode, eleven were excised with

maintenance of a non-leaky seal. Patches in which electrical noise or a poor seal interfered substantially with possible interpretation of oscilloscope deflections were terminated prior to completion of the protocol (Table 2).

4-2.1. Presence of ORCC-Like Channel in IB3-1 Cell Line

Nine of the thirteen patches (69%) were silent patches, demonstrating no channel activity detectable grossly or by analysis with the Pclamp6 program. Although very small channels may have been present, activity was not sufficient to be resolvable by the methods employed. However, the remaining four patches did demonstrate a large outwardly rectifying chloride current. Importantly, this channel was only apparent after the patch had initially been exposed to a depolarizing voltage of at least +70mV. Visual inspection of the amplitude of the channels present in these four (31%) remaining patches suggests that these channels are not CFTR chloride channels. The amplitude, mechanism of activation, behavior after excision, and response to addition of ATP suggests that deflections observed in these active patches represent ORCC events (Figure 4).

Channels present in these four patches had a large amplitude consistent with the reported conductance of 20-30pS for ORCC channels. Moreover, the requirement of a depolarizing voltage of at least +70 mV to activate the channel in each of the four patches also supports the identification of this large channel as ORCC. Of note, this channel remained active at lower voltages after the initial large depolarizing voltage (Figure 5). In addition, unlike CFTR channel activity which reliably decreases after excision due to a requirement for a renewable supply of ATP to maintain channel activity, none of the four

Table 2

Summary of Data Involving IB3-1 Cell Line

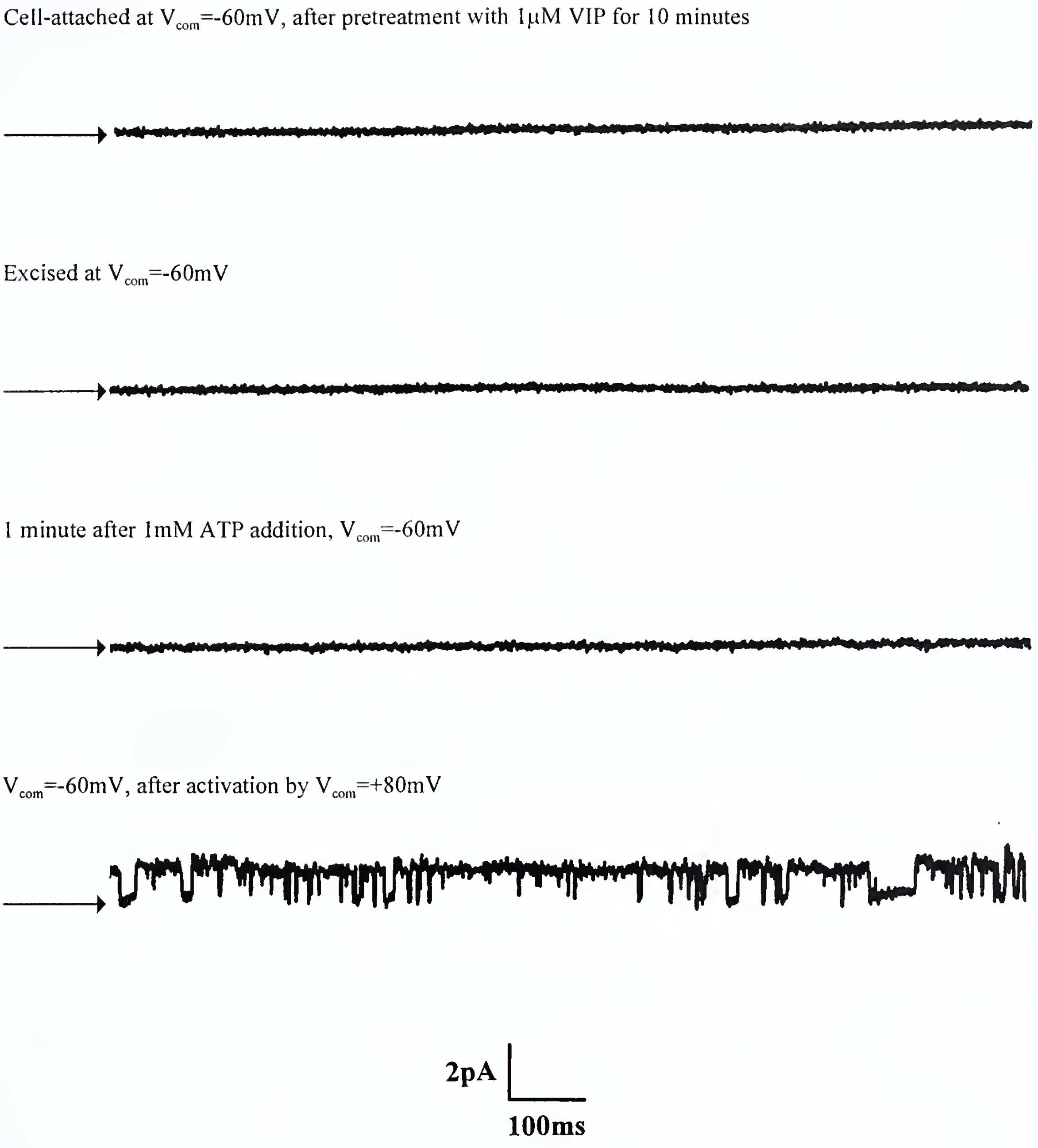
Totals

Number of patches	13
Number of patches without chloride channel activity	9
Number of patches with chloride channel activity	4
Percent of patches with any channel activity	31%
Number of patches with other channel activity	4
Number of patches pretreated with VIP	13
Number of patches pretreated with VIP demonstrating chloride channel activity	4

Of Patches Demonstrating Chloride Channel Activity (n=4)

Channel activity observed in cell-attached mode	3
Channel activity observed in excised mode	3
Rundown evident after patch excision	0
Number of patches treated with ATP	3
Number of patches treated with ATP demonstrating change in channel activity	0
Number of patches demonstrating possible ORCC	4
Percent of patches demonstrating possible ORCC	31%

Note: Previous studies of IB3-1 cells conducted in the absence of VIP demonstrate activation of ORCC only with large depolarizing voltages. No chloride conductance was apparent after manipulation of PKA-regulated mechanisms (Personal communication with Marie E. Egan, M.D.)(Egan, et al., 1992)(Egan, et al., 1995).



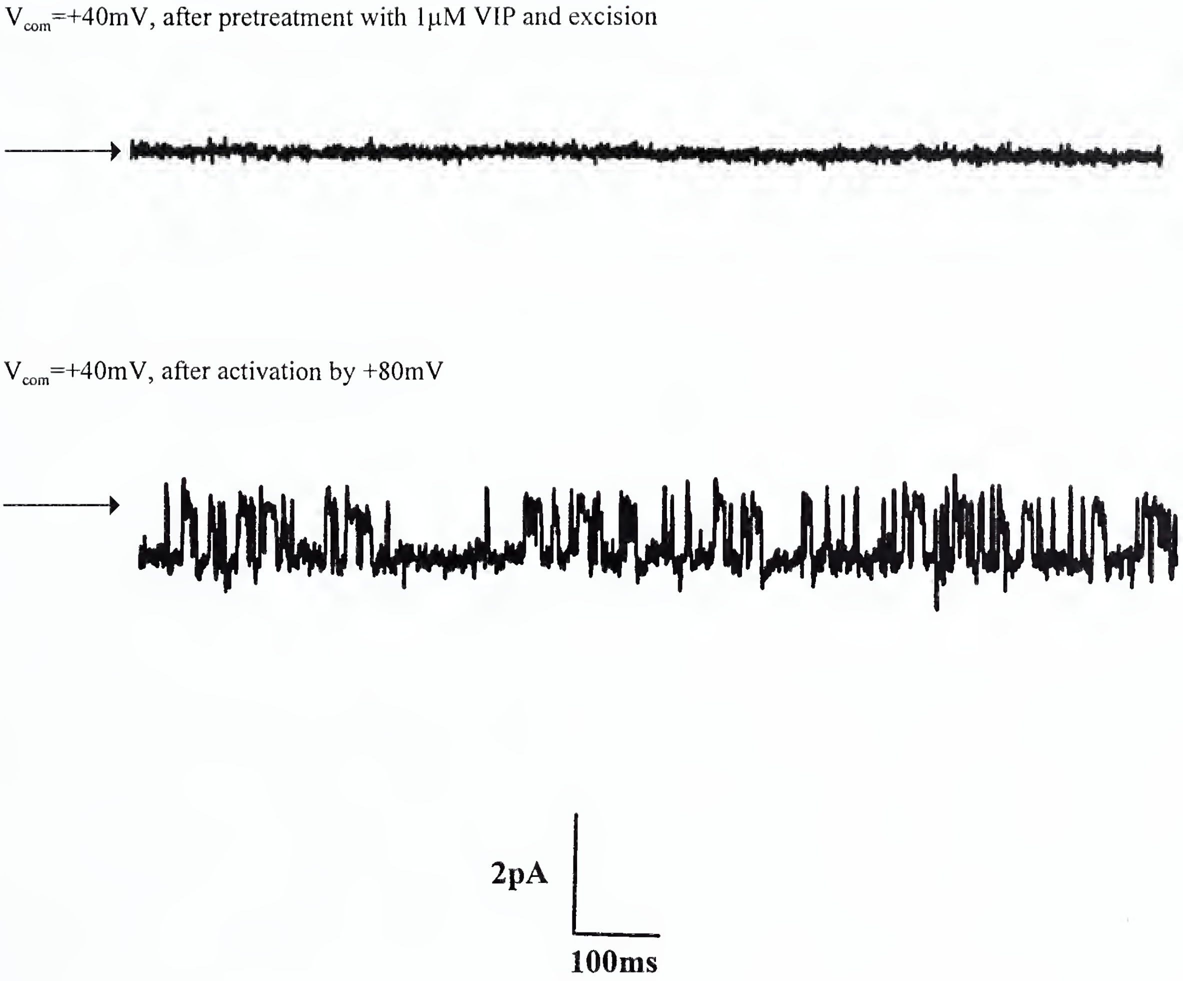
Cell-attached at V_{com}=-60mV, after pretreatment with 1μM VIP for 10 minutes

Excised at V_{com}=-60mV

1 minute after 1mM ATP addition, V_{com}=-60mV

V_{com}=-60mV, after activation by V_{com}=+80mV

Figure 4. Chloride channels in CF airway epithelial cells (IB3-1). Cells from the IB3-1 cell line were pretreated with 1μM VIP for 10 minutes prior to seal formation. The failure of VIP activation of chloride channels in this patch was monitored for 5 minutes in both the cell attached and excised modes. Addition of 1mM ATP to the cytoplasmic aspect of the membrane after excision failed to elicit chloride channel activity. Outwardly rectifying chloride channels were observed after activation by depolarizing voltage. V_{com} is holding potential. V_{mem} equals $-V_{com}$. Upward deflection represents chloride channel opening. Arrows indicate the closed state. Data were digitized at 1kHz and filtered for display at 500Hz. Please note scale where 8mm=2pA.



V_{com}=+40mV, after pretreatment with 1μM VIP and excision

V_{com}=+40mV, after activation by +80mV

Figure 5: Outwardly rectifying chloride channel at low voltage in CF airway epithelial cells (IB3-1). Cells from the IB3-1 cell line were pretreated with 1μM VIP for 10 minutes prior to seal formation. Although no channels were apparent in either the cell-attached or excised modes initially, activation by depolarizing voltage elicited outwardly rectifying chloride channel activity. This channel remained active at voltages of +40 mV or less. V_{com} is holding potential. V_{mem} equals $-V_{com}$. Downward deflection represents chloride channel opening. Arrows indicate the closed state. Data were digitized at 1kHz and filtered for display at 500Hz. Please note scale where 16mm=2pA.

patches with evident channels in the IB3-1 cell line demonstrated “rundown” after channel excision.

Application of 1mM ATP to the solution bathing patches (n=3) that demonstrated no channel activity did not effect any change in apical current. This tactic is often successful in “reviving” CFTR channel activity in patches that have rundown after excision. However, as demonstrated in Figure 4, addition of ATP to the bath solution of the IB3-1 cell line brought about no perceptible difference in activity level compared with prestimulation levels.

4-2.2. Presence of Nonspecific Cation Channel in IB3-1 Cell Line

In four of thirteen patches (31%) a rare small conductance channel was observed which appeared to be consistent with a nonspecific cation channel (Figure 6). Within this cell type, as well as in other epithelial cell lines, a nonspecific cation channel ranging from 10-20pS has been reported previously, as has a smaller cation conductance (5-10pS) activated by stretch. It is unlikely that this channel represents a CFTR chloride channel, given its generally lower mean conductance, and the apparent lack of ATP or PKA dependence. However, because ionic substitution experiments were not performed, the precise nature of this observed channel remains uncertain.

4-3. S9 Cell Line

A gigaseal was successfully obtained and held in twelve patches of the S9 cell line, which was created by transfecting the IB3-1 line with wild-type CFTR. Each of

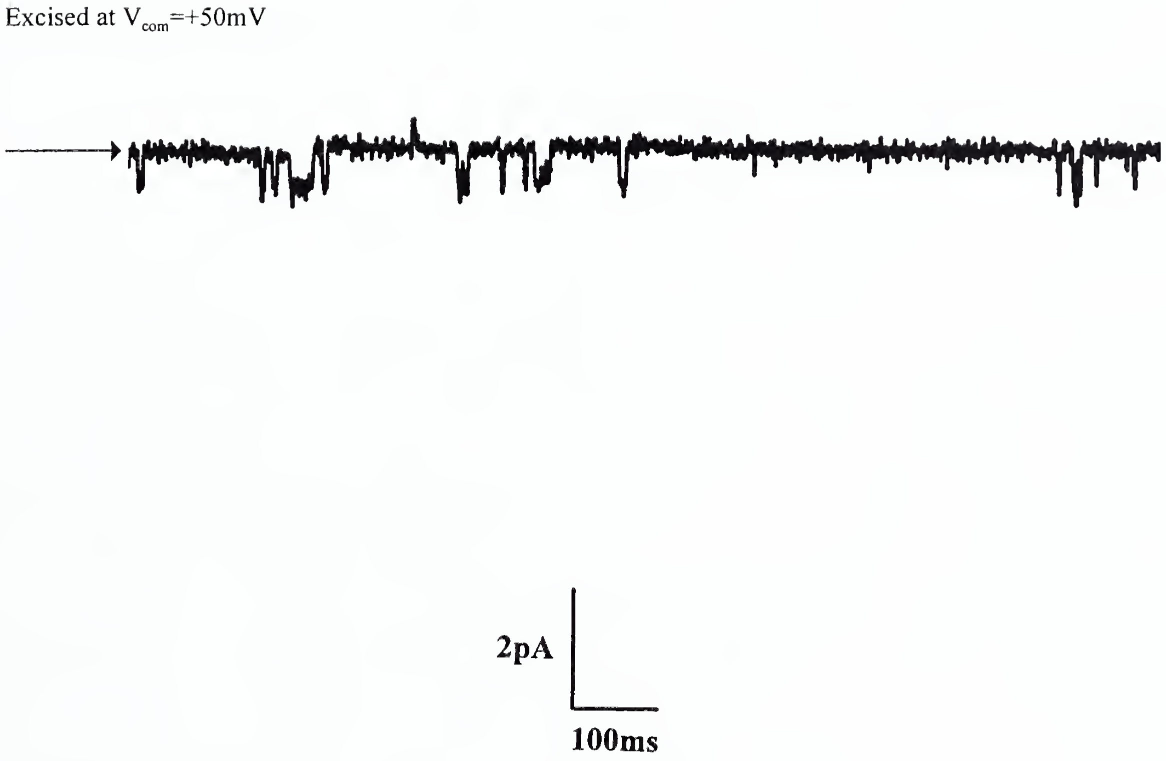


Figure 6: Presence of low conductance channel in CF airway epithelial cells (IB3-1). Cells from the IB3-1 cell line were pretreated with 1μM VIP for 10 minutes prior to seal formation. Rare channel openings were observed in the excised mode in four patches. V_{com} is holding potential. V_{mem} equals $-V_{com}$. Downward deflection represents channel opening. Arrows indicate the closed state. Data were digitized at 1kHz and filtered for display at 300Hz. Please note scale where 16mm=2pA.

these cells had been pretreated with 1μM VIP solution for ten minutes prior to seal formation. In contrast to the IB3-1 cells, ten of these twelve patches (83%) demonstrated channel activity and two were silent patches. Like patches from the IB3-1 line, patches underwent examination throughout the spectrum of holding potentials in both the cell-attached and excised modes. All of the ten patches demonstrated activity in both the cell-attached and excised modes (Table 3).

4-3.1. Presence of CFTR-Like Channel in S9 Cell Line

Unlike the IB3-1 cell line, patches from S9 cells revealed channels with functional characteristics representative of CFTR chloride channels. The mean conductance of the channels observed in these ten samples, calculated from the slope of the linear regression obtained from plotting mean current versus potential (Figure 7), is 10.4pS±0.39pS, which is consistent with the expected conductance reported for CFTR of 8-10pS. Also, as predicted for CFTR, channel activity ran down after patch excision in eight patches (80%) initially displaying oscilloscope deflections (Figure 8). In the two remaining active patches in which rundown was not documented, the seal broke after channel activity similar to that present in the other eight patches was observed, but before rundown occurred. It is likely that these channels also would have exhibited a decrease in activity had the seal of the patch remained stable.

Moreover, in the patches exhibiting rundown after excision, 75% (3/4) of patches treated with 1mM ATP demonstrated a burst of channel activity within three minutes of addition of ATP, which is consistent with CFTR chloride channels (Figure 8).

Table 3

Summary of Data Involving S9 Cell Line

Totals

Number of patches	12
Number of blank patches	2
Percent of patches with channel activity	83%
Number of patches pretreated with VIP	12
Number of patches pretreated with VIP demonstrating channel activity	10

Of Patches Demonstrating Chloride Channel Activity (n=10)

Channel activity observed in cell-attached mode	8
Channel activity observed in excised mode	10
Rundown evident after patch excision	8
Number of patches treated with ATP after rundown	4
Number of patches treated with ATP demonstrating restoration of channel activity	3
Number of patches demonstrating possible ORCC	5
Percent of patches demonstrating possible ORCC	50%

Note: Previous studies of S9 cells conducted in the absence of VIP demonstrate that CFTR chloride channel activity is seen only when cells are stimulated with agents that increase cAMP (forskolin, IBMX) or when excised patches are exposed to PKA and ATP. ORCC is activated either by PKA and ATP addition or by large depolarizing voltages (Personal communication with Marie E. Egan, M.D.)(Egan, et al., 1992)(Egan, et al., 1995).

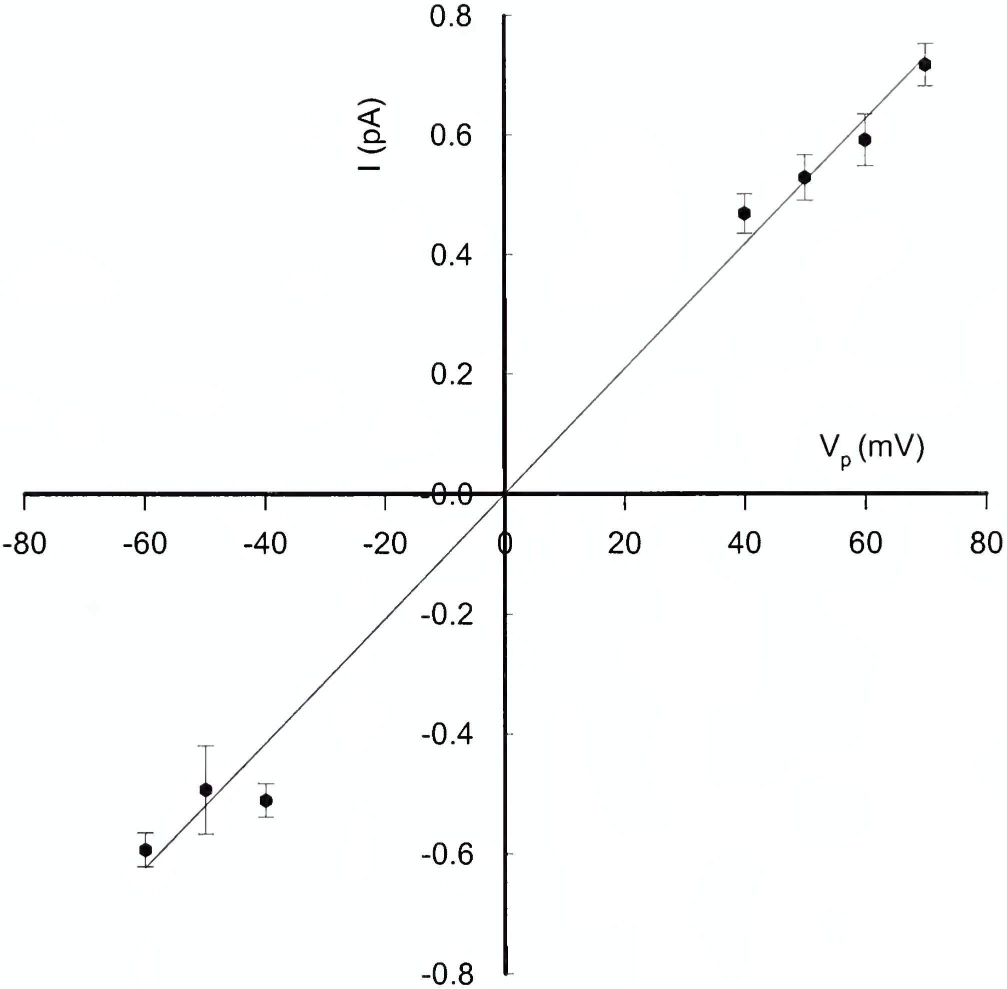
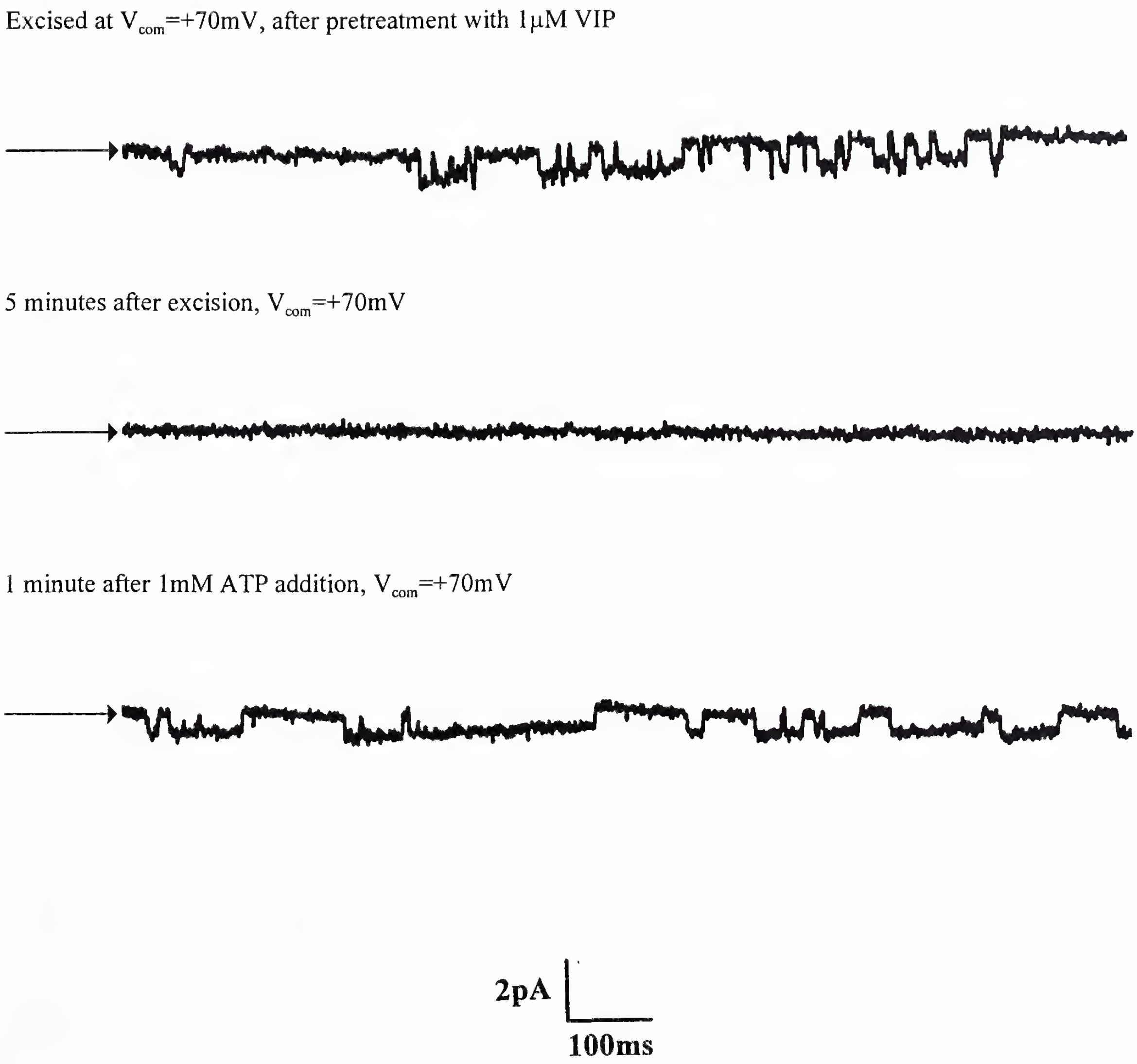


Figure 7. I-V relationship of low conductance channels in S9 cells pretreated with VIP. This figure demonstrates the I-V relationship for low conductance channels in excised inside-out patches bathed in symmetrical chloride solutions $[Cl^-]_i = [Cl^-]_o$ = 150mM (n=10). Patches were excised from S9 cells pretreated with 1μM VIP. The I-V relationship was linear over the voltage range studied. The mean conductance was 10.4pS ±0.39pS. The solid line represents a linear regression fit to the data.



Excised at V_{com}=+70mV, after pretreatment with 1μM VIP

5 minutes after excision, V_{com}=+70mV

1 minute after 1mM ATP addition, V_{com}=+70mV

Figure 8: Activation of low conductance chloride channel in "complemented" IB3-1 cells (S9). Cells were pretreated with 1μM VIP for 10 minutes prior to seal formation. Initially, low conductance channel activity is observed. Rundown of low conductance chloride channel occurred within 5 minutes of patch excision. Addition of 1mM ATP to the cytoplasmic surface of the patch resulted in restoration of low conductance channel activity. V_{com} is holding potential. V_{mem} equals $-V_{com}$. Downward deflection represents chloride channel opening. Arrows indicate the closed state. Data were digitized at 1kHz and filtered for display at 500Hz. Please note scale where 8mm=2pA.

4-3.2. Presence of ORCC-Like Channel in S9 Cell Line

In addition to the smaller, presumed CFTR chloride channel, five of ten (50%) patches exhibited a larger channel suggestive of ORCC (Figure 9). Moreover, eruption of activity invariably coincided with instigation of a high depolarizing voltage of at least +70mV. These channels were not present at voltages less than ±70mV unless the patch had first been exposed to a depolarizing voltage greater than or equal to +70mV. This channel did not demonstrate rundown in any of the five patches, and thus, the affect of added ATP on ORCC activity was not specifically investigated. However, addition of ATP (1mM) following rundown of the small conductance channel did not result in a concurrent increase in ORCC activity.

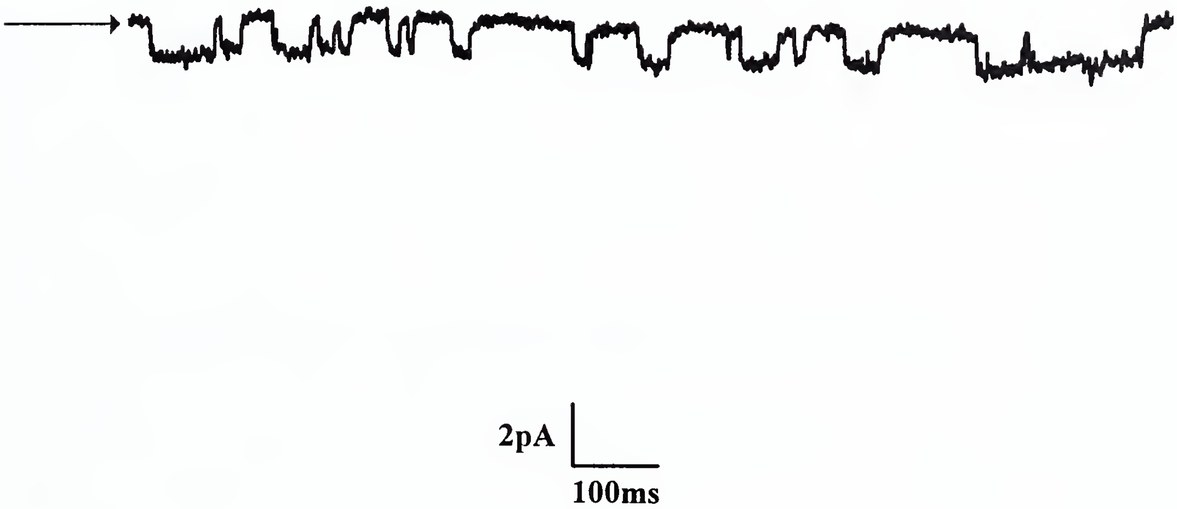
4-3.3. Patches Without Channels in S9 Cell Line

Two of the total twelve patches were silent. Channel activity was absent in both the cell attached and excised modes, and did not appear with ATP addition or depolarization (Figure 10). It is likely, therefore, that these two patches contained no channels in the cell membrane sampled.

4-4. Statistical Analysis

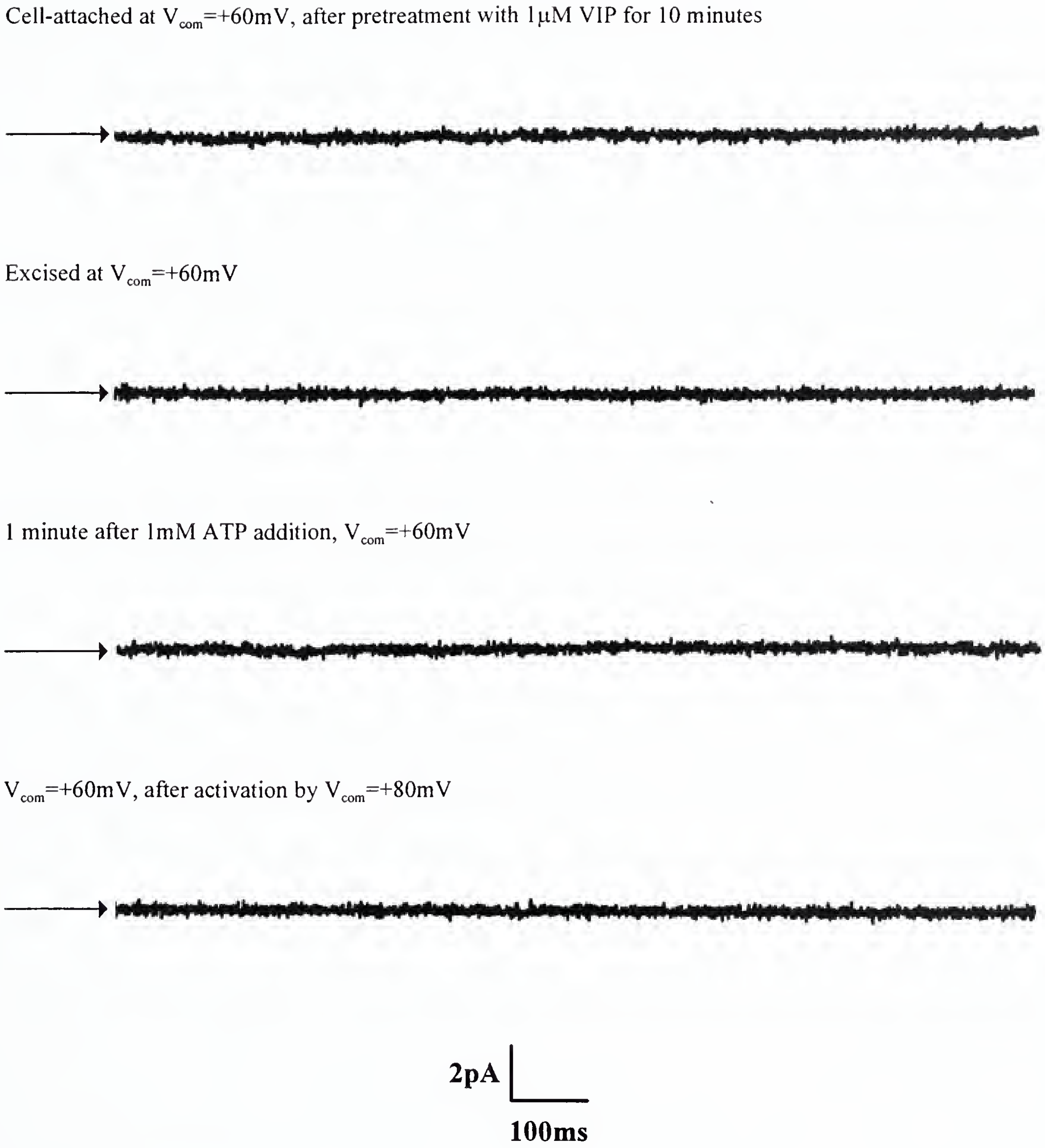
Due to the small sample size of patches analyzed for each cell line, the Fischer exact test was employed to determine the significance of any observed differences between cell lines in the presence of channels in the apical membranes. Presence of CFTR-like chloride currents in the S9 cell line, but not in the IB3-1 cell line produced a P

value of 0.00002. Presence of ORCC-like chloride currents in both the S9 and IB3-1 cell lines produced a P value of 0.6882.



V_{com}=+50mV, after activation by V_{com}=+80mV

Figure 9: Outwardly rectifying chloride channel in "complemented" IB3-1 cells (S9). Cells from the S9 cell line were pretreated with 1µM VIP for 10 minutes prior to seal formation. In addition to the linear low conductance channel (not demonstrated in this figure), a larger outwardly rectifying channel was observed, which was present only after activation by depolarizing voltage. V_{com} is holding potential. V_{mem} equals $-V_{com}$. Downward deflection represents channel opening. Arrows indicate the closed state. Data were digitized at 1kHz and filtered for display at 500Hz. Please note scale where 8mm=2pA.



Cell-attached at V_{com}=+60mV, after pretreatment with 1μM VIP for 10 minutes

Excised at V_{com}=+60mV

1 minute after 1mM ATP addition, V_{com}=+60mV

V_{com}=+60mV, after activation by V_{com}=+80mV

Figure 10: Silent patch in "complemented" IB3-1 cells (S9). Cells from the S9 cell line were pretreated with 1μM VIP for 10 minutes prior to seal formation. The failure of VIP activation of chloride channels in this patch was monitored for 5 minutes in both the cell-attached and excised modes. Addition of 1mM ATP to the cytoplasmic aspect of the membrane after excision failed to elicit chloride channel activity. No outwardly rectifying chloride channels were observed with depolarizing voltage. V_{com} is holding potential. V_{mem} equals -V_{com}. Downward deflection represents channel opening. Arrows indicate the closed state. Data were digitized at 1kHz and filtered for display at 500Hz. Please note scale where 8mm=2pA.

5. Discussion

5-1. Effect of VIP on Chloride Conductance in Airway Epithelial Cells

This study set out to define the mechanism by which VIP increases chloride conductance in airway epithelial cells. We postulated that VIP could act via three unique mechanisms: indirect stimulation through initiation of a second messenger cascade; direct action on chloride channels; or loosening of tight junctions and increase of current through a paracellular path (Figure 11).

A likely pathway included activation of CFTR chloride channels. However, although VIP is known to increase cAMP intracellularly and research has demonstrated CFTR to be a cAMP-dependent chloride channel, one cannot assume that VIP therefore activates CFTR chloride channels through cAMP. As outlined in this paper, several apical chloride channels exist in epithelial cells, at least two of which -- CFTR chloride channels and ORCC -- are cAMP-dependent and regulated by CFTR. Characterization of the chloride current is a first necessary step in pinpointing the mechanism of VIP current activation. A second crucial component in attributing the observed increase to CFTR is to demonstrate that an identical current is not observed upon stimulation with VIP in cells that do not contain the CFTR chloride channel. This study provides evidence that the mechanism of VIP augmentation of chloride current relies on activation of CFTR. However, the data does not unequivocally support or contradict the role of cAMP as the second messenger involved in transduction of the initiating chemical signal.

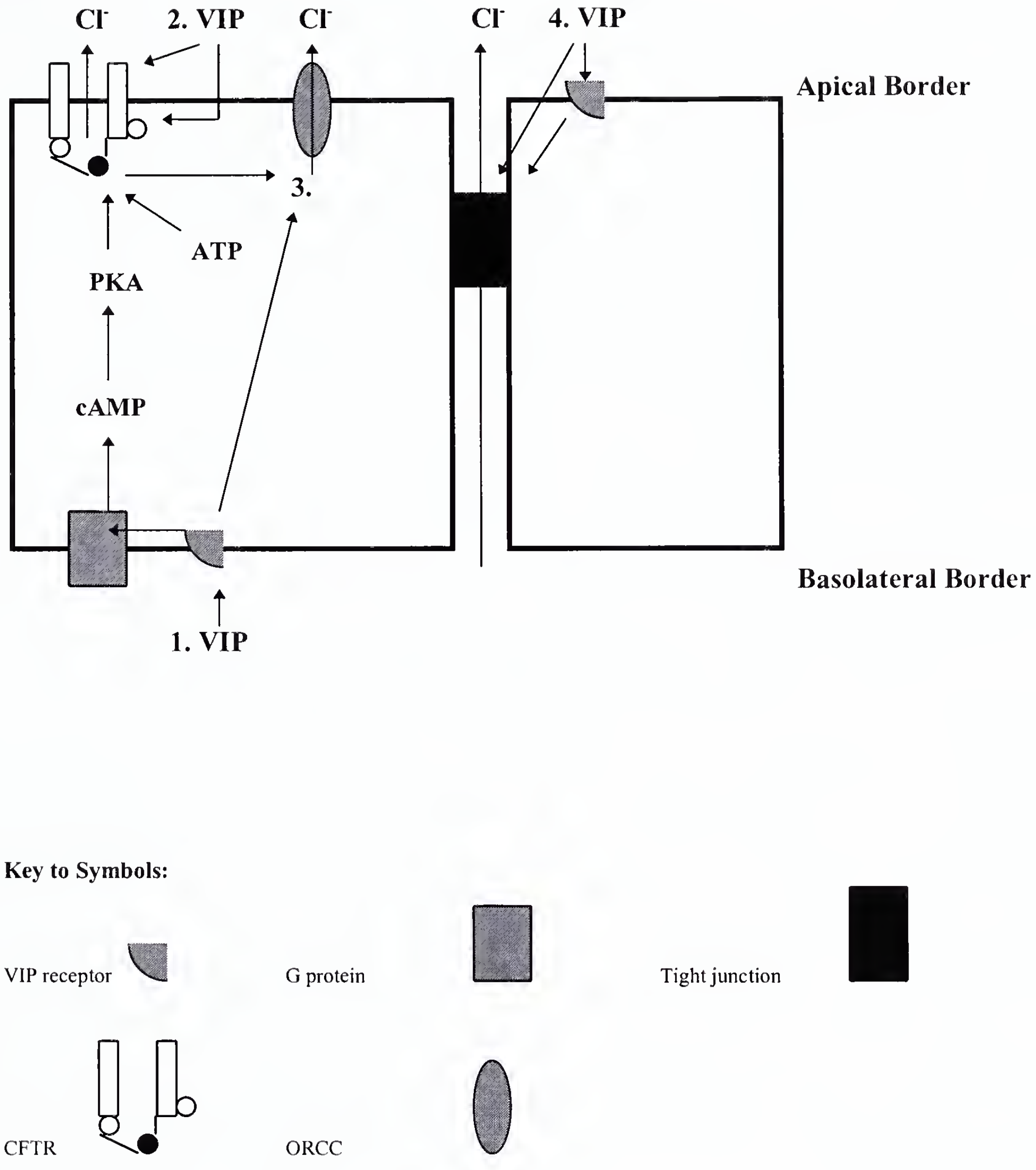


Figure 11. Possible Mechanisms of VIP Stimulation of Chloride Secretion in Airway Epithelia.

1. VIP activates intracellular cascade, which subsequently activates CFTR chloride channels.
2. VIP acts directly on the extracellular or intracellular aspect to stimulate CFTR chloride channels.
3. VIP activates ORCC either indirectly through initiation of intracellular cascade and regulation by CFTR, or directly.
4. VIP acts indirectly through receptors or directly on the epithelial surface to uncouple tight junctions, thus increasing chloride permeability of the layer.

5-2. VIP Indirectly Activates CFTR Chloride Channels

Given understanding of the independent mechanisms of VIP and CFTR, one possible mechanism of increased apical chloride current involves stimulation of CFTR by an increase in cAMP brought about by VIP. Presuming the concentration of VIP was sufficient and that VIP properly activated membrane receptors, all S9 cells pretreated with VIP experienced an increase in intracellular cAMP. In 83% of patches examined from these cells, a 10.4pS±0.39pS current appeared that corresponds in magnitude and direction of flow to that expected for chloride currents generated uniquely by CFTR. This channel has no intrinsic activity at baseline; that is, CFTR demonstrates no chloride current unless it is stimulated by exogenous addition of cAMP or PKA with ATP, or by another agent that supports endogenous supply of cAMP from adenyl cyclase, such as forskolin, IBMX, or a B-adrenergic agonist. Likewise, VIP is an agent capable of increasing cAMP. Thus, a possible scheme might involve VIP-induced generation of cAMP, with consequent phosphorylation of CFTR by PKA and increase in apical chloride current.

Such a model in which VIP activates CFTR is bolstered by two other findings significant for CFTR-generated chloride flow in S9 cells. First, the small linear chloride conductance observed in the cell-attached mode, which has conductance of 10.4pS±0.39pS when excised, ran down to prestimulation levels upon excision in eight of the ten patches with documented channel activity. In CFTR chloride channels, rundown after excision occurs in part when the R domain is dephosphorylated by specific phosphatases near the detached cell membrane and is not rephosphorylated due to the

absence of PKA in the vicinity of the channel. Channel activity is restored by addition of the catalytic subunit of PKA and ATP to the bathing solution. A second pertinent finding is the restoration of the identified 10.4pS±0.39pS current in three of four patches treated with ATP after rundown. Depletion of ATP in the bathing solution after excision is the causative factor in channel rundown observed in CFTR channels. Resurgence of the previously documented current reflects resupply of nucleotide triphosphate for both binding and hydrolysis necessary for channel opening. Other chloride channels recognized in the apical membrane of airway epithelial cells do not exhibit this characteristic pattern of rundown and restoration of current with ATP addition.

In addition, the overwhelmingly different response to VIP observed in S9 cells compared with IB3-1 cells suggests that VIP does activate a pathway whose end-target is stimulation of CFTR. While the small conductance channel appeared in 83% of S9 patches pretreated with VIP, none of the thirteen IB3-1 cells pretreated with VIP manifested a similar channel. This disparity represents a statistically significant difference with $P=2x10^{-5}$. The only genetic dissimilarity between these two cell lines is the presence of CFTR in the S9 cells. In fact, the S9 cell line is derived from the IB3-1 cell line: cells obtained from a CF-affected boy (IB3-1 cell line) were transfected with wild-type CFTR to create the S9 line, the only distinguishing characteristic of which would be the presence of the CFTR gene product.

5-3. VIP Directly Activates CFTR Chloride Channels

Another possible pathway is that VIP acts directly on chloride channels without an intervening second messenger. While the data on conductance and behavior does suggest activation of a CFTR chloride channel, the path of activation could be direct rather than indirect. Although VIP demonstrably increases cAMP in cells, activation of a second messenger cascade, ostensibly designed to magnify the response in physiologic systems, may be bypassed in a system in which fine-tuning of the current is desired. Amplification may not be the goal. Under this rubric, VIP, which is not permeable to the membrane, would activate the channel by binding directly to it on the extracellular surface. Alternatively, VIP could somehow gain access to the cytosolic side of the channel by movement through an unidentified transporter.

If VIP acts directly on the extracellular surface of the channel, one might expect to see an initial burst of activity corresponding to association of VIP with the channel. Provided this connection involved consumption of VIP, one might also see rundown of channel activity as the supply of VIP was depleted. While rundown was observed in patches that demonstrated the VIP-stimulated channel, the pattern of rundown argues against direct stimulation by VIP. Rundown only occurred in patches after excision, and, in fact, was correlated with excision. If rundown were secondary to depletion of VIP, then a decrease in conductance should have been observed in both cell-attached and excised modes as the supply of VIP in the bath solution was diminished. *In vivo*, VIP is rapidly degraded by extracellular and membrane-associated enzymes. Although these experimental conditions did not recreate the milieu in which this reaction might conceivably occur *in vivo*, it seems unlikely that rundown would occur only and reliably

with excision. If extracellular stimulation is the mode of action of VIP in human airway tissue, then one might reasonably expect to see rundown in the cell-attached mode as a natural component of channel gating. Rundown in the cell-attached mode did not occur in any of the ten patches with this channel.

If rundown were secondary to depletion of VIP, one would expect to see restoration of activity with addition of VIP after rundown. This maneuver was not explored in this protocol. However, the restoration of activity with ATP, although not precluding direct VIP activation, does suggest CFTR is phosphorylated, perhaps through the proposed cAMP and PKA pathway.

Factors argue strongly against direct activation of VIP on the cytosolic surface of the channel. First, and perhaps most convincing, is that VIP does not cross the cell membrane. One must postulate an as yet unidentified transporter by which VIP could gain access to the cytosolic side. Alternatively, if a means of access to the cytosolic side was lost in the *in vitro* setting, one might observe channel activity after excision once the cytosolic facet of the channel was exposed to VIP in the inside-out patch. In these samples, however, channels were apparent in both cell-attached and excised modes.

Second, if VIP acted on the cytosolic aspect of the channel, an appreciable delay as VIP crossed the membrane prior to binding would not be surprising. In all of the S9 patches demonstrating channel activity with VIP stimulation (n=10), that activity was apparent immediately upon seal formation. However, one must allow that the time allotted for pretreatment with VIP may have been sufficient for transport across the membrane to occur.

5-4. VIP Increases Chloride Conductance through a Paracellular Pathway

Another possible mode for an increase in chloride flow from the basal to the apical aspect of epithelial cells is through a paracellular, rather than transcellular, path. This formulation involves binding of VIP to receptors to stimulate loosening of tight junctions between epithelial cells. This is the least likely mechanism of the chloride conductance observed in these experiments. If this were the mechanism of action, it would not be revealed by an increase in current observed in the experimental setup we employed. Ion flow between cells would not be witnessed during recordings of patches of membrane from those cells. Thus, even if VIP does have an effect mediated through a paracellular path, it also has the effect reported here attributable to activation of apical CFTR chloride channels.

5-5. VIP Activates ORCC

The apical membrane of the epithelial cells studied contains a second potentially cAMP-regulated channel, the ORCC. Knowing that ORCC can be activated by cAMP, one can construct a model in which VIP increases chloride conductance through ORCC by augmentation of intracellular cAMP. If cAMP is the path by which VIP stimulates chloride flow, then this channel could also serve as the end target of VIP stimulation.

However, ORCC requires the presence of CFTR in the membrane to be cAMP-activated. If VIP modulates chloride conductance through cAMP, IB3-1 cells should display fewer activated ORCC than S9 cells due to the lack of CFTR in the IB3-1 cell line. Analysis of both cell lines showed that 31% of IB3-1 patches versus 50% of S9

patches demonstrated ORCC channels. In contrast to the difference in CFTR chloride channel activity, there is no difference (P=0.6882) in the appearance of ORCC.

Arguing against cAMP as the mediator of VIP and ORCC as a possible end-target is the lack of a temporal association between addition of VIP to either S9 or IB3-1 cells and appearance of channel activity. While the smaller, 10.4pS±0.39pS current appeared in S9 cells pretreated with VIP immediately upon seal formation, the larger current was not observed in any patch (S9 or IB3-1) until after initial depolarizing voltages of at least +70mV. This is surprising because one would expect to see an increase in PKA-dependent ORCC activity corresponding to CFTR activity in the S9 cells, without a requirement for voltage activation.

The sum of the data on ORCC activation in both cell lines does not support a mechanism in which VIP increases chloride conductance via cAMP activation. In the S9 cell line, PKA regulation of ORCC is normal, and one should therefore see an increase in ORCC activity above that observed in the IB3-1 cell line if VIP acts through cAMP. One explanation is that the number of observations obtained in this study is too small to demonstrate a statistically significant difference in ORCC activation between the two cell lines. That is, ORCC is in fact activated in the S9 cell line by VIP mediated increase in cAMP. Because the kinetics of ORCC channels stimulated by the cAMP pathway differ from those stimulated by voltage activation, kinetic analysis of single channel patches would provide a means to delineate the mechanism of ORCC activation. Although single channel data was not obtained in these samples, future studies could examine the kinetics of the ORCC channels observed in these two cell lines to clarify whether VIP is

responsible for channel activation. Alternatively, VIP may act directly on CFTR as outlined above, or it may elicit activation of CFTR via cAMP in a manner which uncouples CFTR regulation of ORCC.

5-6. Therapeutic Implications of VIP Activation of an Apical Chloride Channel

In cystic fibrosis severity of disease directly correlates with the amount of chloride ion effectively secreted across the apical membrane of various epithelia. In people with CF in whom VIP innervation may be abnormal, discovering ways to increase putative VIP-induced chloride channels would clearly have little value. However, in patients in whom VIP innervation is intact, increased chloride current mediated through VIP stimulation could present an attractive therapeutic option that utilizes physiologic pathways to treat this systemic disease, whether VIP acts indirectly through a cAMP-dependent mechanism or directly on CFTR chloride channels.

The data in this study strongly suggest that VIP increases apical chloride conductance in airway epithelial cells by stimulating CFTR chloride channels. Whether VIP acts on CFTR directly, or through increases in intracellular cAMP, remains unresolved. If, in fact, VIP is a member of the family of protein agonists that stimulate CFTR by increasing cAMP (i.e., methylxanthines, IBMX), then VIP is potentially useful in the multitude of CF mutations in which CFTR is made but does not function optimally. These categories of mutations create phenotypes characterized by an absolute insufficient amount of CFTR, protein that spends a disproportionate amount of time in the closed state, or protein that is improperly trafficked to the membrane. The most common CF

mutation, ΔF508, produces a protein without proper trafficking signals. Only a fraction of the necessary CFTR arrives to function at the plasma membrane. Similarly, in treatment of CF patients with adenovirus vector transfection of wild-type CFTR, maintenance of a surfeit of chloride channels in the apical membrane is often a therapeutic problem. Targeting agonists that increase cAMP would maximize stimulation of CFTR present in the plasma membrane, ensuring that chloride secretion, even in a cell with reduced numbers of chloride channels, proceeds most efficiently.

If, as the data on ORCC activation in this study suggest, VIP stimulates CFTR chloride channels through a mechanism that does not involve cAMP as a second messenger, then the value of VIP in CF may be even greater. By utilizing VIP as a potential CFTR agonist in conjunction with known cAMP agonists, therapies could be designed to fully maximize CFTR stimulation. In patients with CF mutations like those described above, using VIP to augment PKA-regulated chloride channel activity would provide a two-pronged approach to increasing ion movement necessary for airway function.

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